The transcriptional regulation of fruit ripening by low temperature

March 2020

Oscar Witere Mitalo

Graduate School of
Environmental and Life Science
(Doctor's Course)

OKAYAMA UNIVERSITY

Declaration

I declare that this thesis has been composed by myself and that the work has not be submitted for any other degree or professional qualification. I confirm that the work submitted here is my own, except where work which has formed part of jointly authored publications has been included. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

The work presented in Chapter 2.1 was previously published in *The Horticultural Journal* as **Characterization of ripening-related genes involved in ethylene-independent low temperature-modulated ripening in 'Rainbow Red' kiwifruit during storage and on-vine** (https://doi.org/10.2503/hortj.OKD-035) by myself (Oscar Witere Mitalo), William O. Asiche, Yuka Kasahara, Yasuaki Tosa, Willis O. Owino, Eric G. Mworia, Koichiro Ushijima, Ryohei Nakano, and Yasutaka Kubo.

The work presented in Chapter 2.2 was previously published in *Postharvest Biology and Technology* as **Comparative analysis of fruit ripening and associated genes in two kiwifruit cultivars ('Sanuki Gold' and 'Hayward') at various storage temperatures** (https://doi.org/10.1016/j.postharvbio.2018.08.017) by myself (Oscar Witere Mitalo), William O. Asiche, Yuka Kasahara, Yasuaki Tosa, Sumire Tokiwa, Koichiro Ushijima, Ryohei Nakano and Yasutaka Kubo.

The work presented in Chapter 2.3 was previously published in *Acta Horticulturae* as **Determination of optimum temperature for long-term storage and analysis of ripening-related genes in 'Rainbow Red' kiwifruit** (https://doi.org/10.17660/ActaHortic.2018.1218.71) by myself (Oscar Witere Mitalo), Sumire Tokiwa, Yuka Kasahara, Yasuaki Tosa, Yuki Kondo, William O. Asiche, Ikuo Kataoka, Katsuhiko Suezawa, Koichiro Ushijima, Ryohei Nakano and Yasutaka Kubo.

The work presented in Chapter 2.4 was previously published in *Frontiers in Plant Science* as **Low temperature storage stimulates fruit softening and sugar accumulation without ethylene and aroma volatile production in kiwifruit** (https://doi.org/10.3389/fpls.2019.00888) by myself (Oscar Witere Mitalo), Sumire Tokiwa, Yuki Kondo, Takumi Otsuki, Ivan Galis, Katsuhiko Suezawa, Ikuo Kataoka, Anh T. Doan, Ryohei Nakano, Koichiro Ushijima and Yasutaka Kubo.

The work presented in Chapter 3 was previously published in *Postharvest Biology and Technology* as **'Passe Crassane' pear fruit (*Pyrus communis* L.) ripening: Revisiting the role of low temperature via integrated physiological and transcriptome analysis** (https://doi.org/10.1016/j.postharvbio.2019.110949) by myself (Oscar Witere Mitalo), Yasuaki Tosa, SumireTokiwa, Yuki Kondo, Azimullah Azimi, Yuko Hojo, Takakazu Matsuura, Izumi C. Mori, Ryohei Nakano, Takashi Akagi, Koichiro Ushijima and Yasutaka Kubo.

……

Oscar Witere Mitalo

Acknowledgements

I would like to express my appreciation to everyone who has helped me to accomplish this doctorate research. In particular, I would like to thank my supervisors Prof. Yasutaka Kubo, Dr. Koichiro Ushijima and Dr. Takashi Akagi for being positive, supportive and knowledgeable supervisors throughout this research. I greatly appreciate all of the suggestions and support you have given me over the last years. To Prof. Kubo, thank you so much for being instrumental both in my academic and social life, and for your financial support when I would run out of scholarship options. Your generous heart cannot go unnoticed. Honestly, there is no better place I would have completed my doctorate research than in your lab. I would also like to thank Dr. Ryohei Nakano (currently at Kyoto University) for his positive suggestions and criticisms while he was in our lab.

I must also extend a special thanks to everyone who assisted me up to this point. Thank you Prof. Ivan Galis and Prof. Izumo C. Mori at the Institute of Plant Science and Resources for allowing me to use your labs for the analysis of aroma volatiles, soluble sugar profiles and phytohormones, without which this project would not have been feasible. Thanks to both of you, I could boost my knowledge in GC/MS and LC/MS analyses. I would also like to thank Dr. William Asiche, Ms. Yuka Kasahara, Mr. Yasuaki Tosa and the rest of my research group (alias the kiwifruit group) for their unrelenting technical support during the period of conducting this research. Prof. Willis Owino and Prof. Daniel Sila at Jomo Kenyatta University of Agriculture and Technology, thank you for encouragement and for introducing me to the research world.

I would also like to express unending gratitude to the Japanese government Ministry of Education, Culture, Sports, Science and Technology who provided me with the payment of the cost of research, tuition fees and living expenses. The opportunity to pursue further studies in Japan will forever be a landmark in my life.

I must also thank the people of Okayama who made a home for me away from home. Thank you to Ms. Mariko Kawachi, Kenny, Ms. Mutsuko Sekiba and the people of Yakage town for making my stay in Okayama comfortable.

Acknowledgement must also go to my mum Noel Anyona, my brothers Festo and Benson as well as my wife Natsuko. Thank you for always supporting and encouraging me to do better. I would not be the person I am today and could not have achieved this without you. Thank you

to my friends James, Justers, Seng, David Wari, Eva Leventer, Sarah Leventer and Annah for your support.

Above all, thanks to the Almighty God for granting me good health and wisdom that were and continue to be indispensable for the success of this project. “We have come this far with the Lord’s help” (1 Samuel 7:12).

Abstract

Fruit ripening is one of the major biological processes that influence the postharvest life of fruit. While the ripening process renders fruit attractive and palatable for consumption, premature/uncontrolled ripening increases their susceptibility to pathogen attack and may cause problems during postharvest handling. Therefore, a better understanding of the underlying regulatory mechanisms is essential for the prediction and/or control of fruit ripening. Cold storage is the most widely used postharvest technology to extend the postharvest life of many fruit. However, low temperature has been shown to enhance fruit ripening in certain fruit species. This thesis investigated low temperature roles in the transcriptional control of fruit ripening in kiwifruit, European pears and citrus fruit.

Kiwifruit are generally classified as climacteric fruit, which essentially require ethylene for fruit ripening induction. However, previous studies have demonstrated that storage at 4–5 ºC can accelerate fruit ripening and related gene expression independently of ethylene. Despite these findings, low temperature-modulated ripening is relatively unexplored compared to ethylene-dependent ripening, and hence the underlying regulatory mechanisms remain unclear. This thesis shows (through studies in 'Rainbow Red' kiwifruit) that low temperature regulates fruit ripening independently of ethylene via a dose × time-dependent mechanism. Relatively low storage temperatures (5 ºC and 10 ºC) sufficiently induced fruit ripening along with the expression of various associated genes within just 4 weeks, while storage at 15 ºC required 8 weeks to elicit a similar response. By contrast, storage at 22 ºC did not provide an adequate ripening stimulus even after 8 weeks. Fruit of the 'Sanuki Gold' kiwifruit cultivar responded rapidly to low temperature as storage at 5 ºC, 10 ºC and 15 ºC induced significant softening, soluble solids concentration (SSC) increase and titratable acidity (TA) reduction within 28 d. The expression levels of various ripening-related genes such as *AcXET2*, *AcPG*, *AcEXP1*, *AcPMEi*, *AcGA2ox1*, *AcMADS2*, *AcNAC5* and *AcbZIP2* also increased rapidly within 28 d during storage of 'Sanuki Gold' fruit at 5 ºC, 10 ºC and 15 ºC. On the other hand, 'Hayward' fruit responded slowly to low temperature as significant ripening and associated gene expression increase occurred only during storage at 5 ºC and 10 ºC. These results indicated that 'Sanuki Gold' fruit are more sensitive to low temperature than 'Hayward' fruit, which would account for the large disparities in harvest maturity dates and postharvest storability between the two cultivars.

Given the findings that low temperature can modulate fruit ripening independently of ethylene, this thesis sought to establish an appropriate temperature for long-term storage of kiwifruit.

Results showed that storage at 22 ºC maintained high fruit firmness and TA for up to 8 weeks, but disease incidence was incredibly high (~77 %), hence ruling out the possible use of this temperature for long-term storage. At 5 ºC, disease incidence was greatly reduced to < 10 % after 8 weeks although there was a sharp increase to 50 % after 12 weeks. However, significant softening and TA reduction occurred relatively faster within 8 weeks, suggesting that 5 ºC can be appropriate for only short-term storage. Fruit softening and TA reduction progressed very slowly during storage at 2 ºC and the disease incidence rate was maintained at minimal levels, which suggested that it could be suitable temperature for medium-term storage. Interestingly, storage at 0 ºC effectively suppressed both fruit ripening and disease incidence rates for up to 12 weeks, suggesting its potentiality for long-term storage of kiwifruit.

Both ethylene-dependent and low temperature-modulated fruit ripening encompass significant fruit softening and SSC increase. GC/MS analysis of soluble sugar profiles further revealed that sucrose, fructose and sucrose contents increased in both ripening systems. However, kiwifruit that were ripened by low temperature showed no production of aroma volatiles (especially ethyl- and methyl-butanoate), which were exclusively induced during ethylene-dependent ripening. The expression of *AcAAT*, whose homologues have been linked to aroma volatile ester production in many fruit such as apples and melons, increased specifically during ethylene-dependent fruit ripening while the changes during low temperature-modulated ripening were minimal. Other ethylene-specific genes including *AcACS1* and *AcNAC5* also showed no expression changes during low temperature-modulated fruit ripening. Conversely, the expression levels of the low temperature-specific gene *AcMADS2* did not change significantly during ethylene-dependent fruit ripening. These findings clearly demonstrated that ethylene signalling is non-functional during low temperature-modulated fruit ripening, and that ethylene-dependent and low temperature-modulated fruit ripening involve distinct regulatory mechanisms.

European pear fruit, like kiwifruit, are also considered climacteric as fruit ripening changes are primarily driven by ethylene-regulated changes in gene expression. In most cultivars, especially the late-maturing ones such as ‘Passe Crassane’ and ‘Rocha’, low temperature storage is commercially used to induce ethylene production and subsequently, fruit ripening. However, the mechanisms involved in this unique ripening behaviour remain to be seen. In this thesis, we followed the physiological and molecular responses of ‘Passe Crassane’ pears to low temperature and the ethylene analogue, propylene, at various storage temperatures. Fruit at 20 ºC treated with propylene softened to eating firmness (13–21 N) within 9–10 d, with little

changes in endogenous ethylene production (< 0.03 μg kg^{-1} s^{-1}). By contrast, low temperature-treated fruit (0 ºC and 5 ºC for 42 d) produced large amounts of ethylene (1–2 μg kg^{-1} s^{-1}), and rapidly softened to < 5 N after being transferred to 20 ºC. From transcriptomic analyses, we identified 437 differentially expressed genes (DEGs) between propylene-treated and control fruit, which were further augmented by low temperature treatment. On the other hand, the expression patterns of 763 DEGs between 5 ºC vs. 20 ºC was not significantly affected by propylene treatment in non-chilled fruit. To examine low temperature-induced and ethylene-induced pathways separately during chilling, the responses of low temperature-induced DEGs to 1-methylcyclopropene (1-MCP), an ethylene action inhibitor, were assessed. Among the 763 low temperature-induced DEGs, 1-MCP treatment disrupted the expression of 390 DEGs, indicating that they were regulated by low temperature-induced ethylene. Intriguingly, 373 DEGs including transcription factor-related genes such as *PcERF98-like*, *PcATL65*, *PcMYB6-like*, *PcGRP2-like*, *PcTCP7* and *PcMBF1c* were unaffected by 1-MCP treatment, and thus, likely to be influenced by low temperature alone. Therefore, it appears that these low temperature-specific genes play a critical role to activate ethylene biosynthetic genes, and to potentiate ethylene responsiveness of ripening-related genes.

Peel degreening is the most obvious aspect of fruit ripening in citrus fruit species, and it involves the degradation of green-coloured chlorophyll pigments and/or the synthesis of carotenoid pigments of varying colours including yellow, orange or red. Although classified as non-climacteric fruit, ethylene treatment enhances peel colour changes, and this has prompted wide use of the hormone in commercial peel degreening. Earlier studies in lemons, grapefruit, oranges and satsuma mandarins have demonstrated that low temperature can also promote peel degreening both during postharvest storage and on-tree maturation. However, the role of low temperature in this process remains unclear and most studies attribute it to the trace levels of system I ethylene produced by most plants.

In this study, treatments with either ethylene or propylene triggered peel degreening in lemons and satsuma mandarins within 4 d. A single treatment of fruit with 1-MCP (2 uLL^{-1}) for 12 h strongly inhibited ethylene-induced peel degreening in lemons. Peel degreening was also triggered by moderately low storage temperatures (5 ºC, 10 ºC, 15 ºC and 20 ºC) in lemons, and by 10 ºC and 15 ºC in satsuma mandarins after 28–42 d. No appreciable peel colour changes were observed during storage at 25 ºC for lemons, and 5 ºC, 20 ºC and 25 ºC for satsuma mandarins. Surprisingly, repeated treatments with 1-MCP to block ethylene signalling failed to inhibit the accelerated peel colour changes observed at low storage temperatures.

Transcriptome analysis in both lemons and satsuma mandarins revealed that low temperature and ethylene independently regulated genes associated with chlorophyll degradation, carotenoid metabolism, photosystem proteins, phytohormone biosynthesis and signalling, and transcription factors. On-tree peel degreening correlated well with environmental temperature drops, and it coincided with the differential expression of low temperature-regulated genes. In contrast, genes that were uniquely regulated by ethylene showed no significant expression changes during on-tree peel degreening. These findings strongly argue for the hypothesis that low temperature plays a prominent role in regulating natural peel degreening independently of endogenous ethylene in citrus fruit.

Altogether, it is evident from the results of this thesis that contrary to the conventional understanding, low temperature can modulate fruit ripening either independently or in conjunction with ethylene. This conclusion is however limited in the fact that evidences obtained in this thesis are only at the transcriptional level. Advanced research tools such as gene editing via CRISPR/Cas9 techniques and protein assays would enable us to clearly elucidate low temperature perception and signalling pathways in the fruit ripening process. This would in turn boost our ability to control and/or predict fruit ripening, and hence reduce postharvest losses that have posed a constant menace to the global food security situation.

Table of Contents

CHAPTER 1

A general introduction into fruit ripening and its regulation

Preface

Fruit ripening is a process by which the biochemistry and physiology of fruit are developmentally altered to influence their appearance, texture, flavour and aroma (Giovannoni, 2004). From an evolutionary perspective, fruit ripening is seen as a survival strategy used by plants to attract seed-dispersing animals. However, fleshy fruit are predominant sources of fibre, vitamins, water, minerals and various anti-carcinogenic phytochemicals that are required for a healthy human diet (Laura *et al*., 2009; Slavin and Lloyd, 2012), and thus provide unique and critical contributions to food security. Fleshy fruit are considered perishable produce that are subject to decay during postharvest handling, and this can be aggravated by fruit ripening. Premature or uncontrolled fruit ripening especially increases the susceptibility of fruit to pathogen attack and may cause problems during postharvest handling of fruit commodities (Golden *et al*., 2014). Thus, better understanding of the regulatory mechanisms involved in fruit ripening is required to boost our ability to predict and/or control the ripening process in fruit and hence maintain the quality attributes of the fruit during the postharvest handling.

This chapter aims to give a background of our current understanding of the physio-molecular factors that orchestrate fruit ripening. Additionally, this chapter endeavours to enlighten the reader on the progress made so far in understanding the role of low temperature in regulation of fruit ripening and overall fruit physiology. The outcome of researching this background material will be to use this information to identify mechanisms that low temperature may use to stimulate certain fruit ripening aspects.

1.1. The physiology and biochemistry of fruit ripening

Fruit ripening is a complex process involving various changes that can include a colour change, softening, an increase in sugars, a decrease in acidity and release of aroma volatiles (Seymour *et al*., 2014). Fruit ripening-associated changes vary depending on the fruit species and cultivar. However, most fruit exhibit some, if not all, of the above ripening attributes. Each fruit ripening attribute is tightly regulated at the transcriptional level through the up and downregulation of genes encoding various structural and accessory proteins.

1.1.1. Fruit softening

Ripening-associated fruit softening is complex in nature, although it typically involves modifications to the cell wall components, especially the polysaccharides and proteins

(Brummell, 2006; Vicente *et al*., 2007; Tucker *et al*., 2017). Fruit softening during ripening was shown to be a result of the concerted action of various enzymes such as polygalacturonase (PG), pectate lyase (PL), β-galactosidase (β-GAL), pectin esterase (PE), 1,4-β-glucanase, xyloglucan transglycosylase/hydrolase (XTH) and expansin (EXP) (Tucker *et al*., 2017). These enzymes are involved in the depolymerisation, de-esterification and transglycosylation of pectin, cellulose and hemicellulose, and in the disruption of hydrogen bonds that bind the polysaccharides, resulting in cell wall loosening (Brummell, 2006; Vicente *et al*., 2007).

1.1.2. Increase in soluble sugar content

Accumulation of soluble sugars is another important change that is associated with fruit ripening. This has been attributed to the active conversion of starch, the major carbohydrate in most mature fruit, to soluble sugars (sucrose, glucose and fructose) through the action of β-amylase (β-AMY) and α-amylase (α-AMY) (Hu *et al*., 2016; Maria *et al*., 2016; Xiao *et al*., 2018). Soluble sugar accumulation during fruit ripening is also linked to the action of other enzymes such as sucrose synthase (SUS), sucrose phosphate synthase (SPS), and invertases that are involved in sugar interconversion (Kim *et al*., 2015).

1.1.3. Reduction in acidity

The major organic acids in fruit are citrate, malate and ascorbate, and it is widely recognized that their content, especially that of citrate and malate, dramatically decreases during fruit ripening (Osorio *et al*., 2012; Merchante *et al*., 2013). The molecular mechanisms that are involved in acidity reduction during ripening have received much less attention to date. However, it is believed that the ripening-associated reduction of malate and citrate content is due to increased respiratory metabolism of these compounds (Centeno *et al*., 2011).

1.1.4. Colour changes

Colour changes during fruit ripening can occur in the peel and/or flesh, depending on the fruit species and cultivar. In either case, colour change results from the ultrastructural transformation of chloroplasts to chromoplasts (Rodrigo *et al*., 2013), and the degradation of green-coloured chlorophyll pigments (Bramley, 2002; Grassi *et al*., 2013), as well as from the biosynthesis and metabolism of carotenoids with varying colours (Yuan *et al*., 2015; Ohmiya *et al*., 2019). The chlorophyll degradation pathway is well established in higher plants (Hörtensteiner, 2006), and it involves the sequential conversion of chlorophyll pigments to

colourless non-fluorescent derivatives. Carotenoids provide fruit with different colours that range from yellow and pink to deep orange and red depending on the fruit tissue and species. The ripening process is marked by dramatic changes in the content and composition of carotenoids, and the biochemical steps involved have long been established (Nisar *et al*., 2015; Yuan *et al*., 2015).

Apart from chlorophylls and carotenoids, other compounds such as flavonoids also contribute to the colour of many fruit during ripening. Flavonoids, especially anthocyanins, form the major pigments in ripe strawberry (Medina-Puche *et al*., 2014), grape (Castellarin *et al*., 2007), and peach (Ravaglia *et al*., 2013). Flavonoids are responsible for the major red, purple, violet, and blue pigments found in many fruit (Petroni and Tonelli, 2011).

1.1.5. Release of aroma volatiles

Most fruit also produce a large number of aroma volatile compounds as an indicator of fruit ripening. Although the volatile profiles of fruit vary depending on the species, cultivar, ripening stage and postharvest conditions (El Hadi *et al*., 2013), the major groups include alcohols, terpenoids, aldehydes and particularly, esters (Defilippi *et al*., 2009). Volatile esters often represent the major contribution of aroma during ripening in many fruit including kiwifruit, apple, pear and peach (Fellman *et al*., 2000; Ortiz *et al*., 2010). On the other hand, terpenoids such as limonene, S-linalool, valencene and β-pinene are key compounds determining the characteristic aroma of ripe tomato, strawberry and citrus.

1.2. The role of ethylene in fruit ripening regulation

Ethylene is a simple gaseous plant hormone that is made up of two carbons bound to four hydrogen molecules (C_2H_4). Most plant tissues naturally produce ethylene for the regulation of a wide range of growth and developmental processes, as well as for response to environmental stresses (Abeles *et al*., 1992, Lashbrook *et al*., 1998; Schaller, 2012; Khan *et al*., 2017). However, ethylene is widely known for its extreme ripening effect in many fruit, earning it an alias “the ripening hormone”.

1.2.1. Climacteric and non-climacteric fruit ripening

Fruit are broadly classified into two main types (climacteric or non-climacteric) based on their ripening patterns (Paul *et al*., 2012) (Table 1.1). Climacteric fruit ripening is accompanied by

an increase in respiration and a simultaneous rise in ethylene production (Lelievre *et al*. 1997a; Paul *et al*., 2012). The rate of ethylene production during ripening varies greatly among different species and cultivars of climacteric fruit such as tomato, kiwifruit, apple and peach. Most of the ripening-associated changes in climacteric fruit are driven by ethylene-regulated changes in gene expression and enzyme activity (Giovannoni, 2004). The application of exogenous ethylene can also trigger fruit ripening in climacteric fruit (Agar *et al*., 1999). On the other hand, non-climacteric fruit ripening occurs in the absence of any detectable increase in ethylene production (Paul *et al*., 2012).

A major weakness of this classification of fruit is that ethylene has been implicated in the regulation of certain fruit ripening attributes in some classical non-climacteric fruit. Although citrus fruit as considered non-climacteric, ethylene has been shown to trigger fruit ripening-related chlorophyll degradation and carotenoid biosynthesis (Jacob-Wilk *et al*., 1999; Shemer *et al*., 2008). A second weakness of this classification is that some fruit ripening-associated changes in classical climacteric fruit have been shown to be independent of ethylene regulation in tomato and banana (Golding *et al*., 1998; Yokotani *et al*., 2009), or to occur in the absence of any detectable ethylene increase in kiwifruit (Kim *et al*., 1999; Richardson *et al*., 2011).

Table 1.1. Classification of fruit into climacteric and non-climacteric groups

Climacteric	**Non-climacteric**
Tomato, Apple, Mango, Papaya, Banana, Pear, Kiwifruit, Persimmon, Avocado, Peach, Plum, Passion fruit, Guava, Durian, Apricot, Cherimoya	Watermelon, Citrus (orange, grapefruit, lemon, lime etc), Grape, Strawberry, Pumpkin, Squash, Pineapple, loquat, Olive, Pomegranate, Jujube

1.2.2. Regulation of ethylene production

In higher plants, ethylene is biosynthesized through a three-step pathway (Fig. 1.1) involving the conversion of (i) methionine to S-adenosyl-*L*-methionine (SAM) by SAM synthetase, (ii) SAM to 1-amino cyclopropane-1-carboxylate (ACC) by ACC synthase (ACS), and (iii) ACC to ethylene by ACC oxidase (ACO) (Kende, 1993). The key ethylene biosynthetic enzymes are ACS and ACO, both of which are encoded by multigene families (Wang *et al*., 2002; Cara and Giovannoni, 2008). Earlier studies have demonstrated that ACS catalyses the rate-limiting step for ethylene biosynthesis (Kende, 1993; Wang *et al*., 2002). The genes encoding both ACS and ACO have been isolated and structurally characterized, and they are differentially expressed in

various tissues at different stages of development and in response to internal or external stimuli such as ripening, senescence, wounding and pathogen attack (Lelievre *et al*., 1997a; Nakatsuka *et al*., 1998; Bouzayen *et al*., 2010).

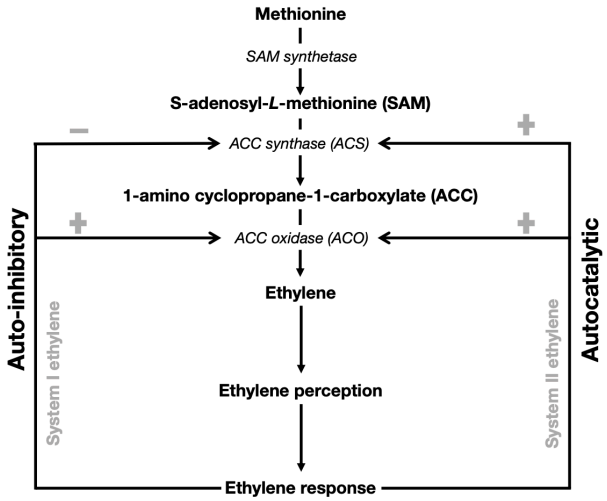


Fig. 1.1. A simplified ethylene biosynthesis pathway in plants showing system I (auto-inhibitory) and system II (autocatalytic) ethylene production. ACO expression and activity are induced by ethylene in both systems but no auto-induction is observed in system I due to absence of ACS activity enhancement. (Modified from Paul *et al*., 2012).

There are two systems of ethylene production that have been defined in plants (McMurchie *et al*., 1972; Kende, 1993). The first one is designated as system I and it is responsible for the basal levels of ethylene production in vegetative tissues and unripe fruit (Barry and Giovannoni, 2007). This system is regulated in auto-inhibitory manner, that is, presence of exogenous ethylene suppresses further biosynthesis of ethylene (Kende, 1993) (Fig. 1.1). The second system is referred to as system II and it is responsible for the dramatic increase in ethylene production during fruit ripening and floral senescence (Barry and Giovannoni, 2007). System II is regulated in an autocatalytic manner (Fig. 1.1), as exogenous ethylene stimulates its own biosynthesis as well as rapid fruit ripening (Lelievre *et al*. 1997a; Nakatsuka *et al*. 1998; Inaba *et al*; 2007; Yokotani *et al*., 2009). In a broad perspective, climacteric fruit ripening is regulated by system II ethylene whereas non-climacteric fruit produce basal levels of system I ethylene throughout the ripening stages.

The physio-molecular pathways that act to initiate the transition from system I to system II ethylene biosynthesis are largely unknown. One explanation is that the cumulative effects of system I ethylene reach a certain limit where they can induce system II ethylene production (Klee, 2004). The second explanation is that there is a change in ethylene sensitivity during fruit maturation, that is, fruit might become more sensitive to system I ethylene as its development progresses (Barry *et al*., 2000). The third explanation is that system II ethylene

production might be induced by other hormones. Work in peach has shown that increased levels of auxin are required for system II ethylene production via the induction of various *ACS* genes (Tatsuki *et al*., 2013; Pan *et al*., 2015). Additionally, stresses such as wounding, water stress, and disease during fruit development induce ethylene production and this shortens the period required for the onset of fruit ripening (Abeles *et al*., 1992; Nakano *et al*., 2003).

1.2.3. Ethylene perception and signal transduction pathway

Plants perceive ethylene using a family of receptors that are localized on the endoplasmic reticulum (Chang *et al*., 1993; Ju and Chang, 2015). Multiple ethylene receptor complexes have been identified in Arabidopsis and tomato including ethylene receptor 1 (ETR1), ETR2, ethylene response sensor 1 (ERS1) ERS2 and ethylene insensitive 4 (EIN4), all of which function as negative regulators of ethylene action (Ju and Chang, 2015). The ethylene signalling pathway is highly conserved in higher plants (Fig. 1.2). Ethylene binding results in the degradation of these receptors, disrupting its interaction with constitutive response 1 (CTR1). This subsequently activates EIN2 that in turn stabilises multiple families of EIN3 and EIN3-like (EIL) transcription factors (TF). From EIN3 and EILs, the signal is then transmitted to a large group of TFs termed ethylene responsive factors (ERF) that have been shown to modulate specific genes associated with various biological responses such as fruit ripening.

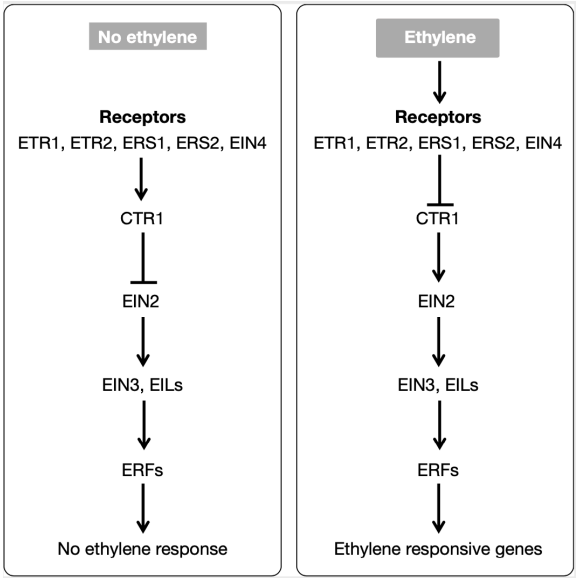


Fig. 1.2. Model for ethylene perception and signaling in plants. Ethylene receptors, together with CTR1 function as negative regulators. In the absence of ethylene, receptors bind to CTR1 which inactivates EIN2 via phosphorylation. Ethylene binding degrades the receptors and subsequently inactivates CTR1 which releases EIN2 that stabilizes and activates EIN3/EILs. The signal is transmitted to ERFs (the primary ethylene target genes) which then modulate various genes (secondary ethylene responsive genes) that are associated with a wide range of biological processes such as fruit ripening.

As stated earlier in section 1.2.1, many fruit ripening-associated changes are regulated by the ethylene signalling pathway especially in climacteric fruit. Ethylene signalling stimulates the expression of various softening related genes in tomato (Sitrit and Bennet, 1998), kiwifruit (Wang *et al*., 2000; Mworia *et al*., 2012), and apple (Ireland *et al*., 2014), among others. Additionally, various genes associated with starch degradation and sugar accumulation have been shown to be influenced by ethylene (Hu *et al*., 2016). Ethylene also modulates ripening-related colour changes through the stimulation of genes associated with chlorophyll degradation and carotenoid metabolism both in climacteric fruit such as tomato (Su *et al*., 2015), and non-climacteric fruit such as citrus fruit (Goldschmidt *et al*., 1993; Rodrigo and Zacarias, 2007; Shemer *et al*., 2008). Works in various fruit species have demonstrated that aroma volatile production during fruit ripening strictly relies on the presence of ethylene signalling (Defilippi *et al*., 2004; Schaffer *et al*., 2007; Pech *et al*., 2008; Atkinson *et al*., 2011).

1.2.4. Propylene is an analogue of ethylene

Earlier studies have identified and characterized several organic compounds that exhibit a similar function as ethylene (Burg and Burg, 1967, Abeles and Gahagan, 1968), among which propylene was identified as the most active ethylene analogue (Table 1.2). Treatment of fruit with propylene triggers ethylene signalling, resulting in autocatalytic ethylene production and accelerated fruit ripening in climacteric fruit. McMurchie *et al*. (1972) effectively used propylene treatment to identify system II ethylene production in banana. Propylene treatment has also long been used to study ethylene-dependent and independent fruit ripening in various fruit including kiwifruit (Antunes *et al*., 2000; Mworia *et al*., 2012), banana (Golding *et al*., 1998; Inaba *et al*., 2007), and tomato (Giovannoni *et al*., 1989). The use of propylene treatment is beneficial because it allows for accurate measurement of endogenous ethylene produced by the fruit sample in question. Therefore, propylene treatments are widely used in this thesis for the main purpose of triggering ethylene signalling.

Table 1.2. Biological activity of ethylene and other unsaturated compounds (Burg and Burg, 1967)

Compound	ppm of gas for half-maximum biological activity
Ethylene	0.1
Propylene	10
Vinyl chloride	140
Carbon monoxide	270
Vinyl fluoride	430
Acetylene	280
Allene	2900
Methyl acetylene	800
1-Butene	27000
Vinyl bromide	1600
Ethyl acetylene	11000
Vinyl methyl ether	10000
Butadiene	500000

1.2.5. 1-Methylcyclopropene (1-MCP) is a competitive inhibitor of ethylene action

A number of molecules including 1-MCP, silver nitrate and 2,5-norbornadiene (NBD) have been shown to counteract ethylene effect by interaction with ethylene receptors (Goren *et al*, 1984; Sisler and Yang, 1984; Sisler and Serek, 1997). Among them, 1-MCP stands out because of its powerful ethylene inhibitory effect and eco-friendly nature (Golding and Singh, 2017). 1-MCP is a synthetic cyclic olefin that binds ethylene receptors with an affinity that is about 10 times higher than that of ethylene (Jiang *et al*., 1999; Golding and Singh, 2017). Unlike ethylene, 1-MCP irreversibly binds ethylene receptors and the resulting 1-MCP-receptor complexes block ethylene perception even in the presence of the hormone (Watkins, 2006; Golding and Singh, 2017). The duration of ethylene insensitivity after 1-MCP treatment depends on the rate of turnover of the 1-MCP-receptor complex, and this varies greatly among different fruit species. For instance, kiwifruit treated with 1-MCP become insensitive to ethylene for about 5 d (Mworia *et al*., 2012). On the other hand, 1-MCP treatment can be severe in pear fruit cultivars as it indefinitely inhibits ethylene-dependent fruit ripening (Villalobos-Acuna and Mitcham, 2008). Since its discovery as an ethylene perception inhibitor, 1-MCP has been extensively used in postharvest handling of fruit and vegetables (Watkins, 2006). In addition, several studies have employed 1-MCP to unravel ethylene-dependent and independent fruit ripening regulation in kiwifruit (Mworia *et al*., 2012; Asiche *et al*., 2018), banana (Zhang *et al*., 2006), papaya (Ergun and Huber, 2004), tomato (Hoeberichts *et al*., 2002), and avocado (Jeong and Huber, 2004).

1.3. Insights into the transcriptional control of fruit ripening

There is growing understanding of how fruit ripening is regulated at the molecular level. Studies in tomato have revealed a number of TFs that bind to and activate various fruit ripening-related genes such as those for ethylene biosynthesis, and specific fruit ripening attributes. This transcriptional regulatory network is evident from a large number of mutations that affect ethylene production leading to defective fruit ripening.

1.3.1. The MADS-box domain

The best studied transcriptional regulator of fruit ripening is the MADS-box family of transcription factors including ripening inhibitor (RIN) and colourless non-ripening (CNR). *RIN* has been shown to bind a large number of fruit ripening related genes (Fujisawa *et al*., 2013), and it is highly up-regulated during fruit ripening in tomato (Vrebalov *et al*., 2002), banana (Elitzur *et al*., 2010), strawberry (Seymour *et al*., 2011), and apple (Ireland *et al*., 2013). Furthermore, the *rin* mutation results in a complete inhibition of system II ethylene production and subsequent fruit ripening (Manning *et al*., 2006; Ito *et al*., 2017). The *Cnr* mutation is epigenetic due to methylation in the promoter of squamosa binding protein (SBP), and it also effectively blocks fruit ripening (Manning *et al*., 2006; Giovannoni, 2007). Additional MADS-box transcription factors that regulate fruit ripening include tomato agamous-like (TAGL) (Itkin *et al*., 2009), and FUL/TDR4 (Bemer *et al*., 2012).

1.3.2. The NAC domain

NAC domain TFs also have a critical role in the regulation of fruit ripening. The first NAC family TF to be characterized in NOR, and like *rin*, *nor* tomato fruit fail to produce system II ethylene and thus fail to ripen normally (Giovannoni *et al*., 1995; Karlova *et al*., 2014). Since then, several other NACs have been shown to modulate ethylene biosynthesis and fruit ripening in various fruit including tomato (Zhu *et al*., 2013; Kou *et al*., 2016), banana (Shan *et al*., 2014), and kiwifruit (Nieuwenhuizen *et al*., 2015).

1.3.3. Ethylene responsive factors

The ERFs belong to the large AP2/ERF multigene family that are known to mediate ethylene-dependent gene expression (Pirrello *et al*., 2012). ERFs have also been shown to play a role in different plant developmental processes, including fruit ripening. Generally, ERFs act downstream of the ethylene signalling, where they directly modulate ethylene-regulated target

genes (Pirrello *et al*., 2012). A large number of ERFs have been associated with ethylene-dependent fruit ripening in many fruit including tomato (Chung *et al*., 2010; Liu *et al*., 2016), kiwifruit (Yin *et al*., 2010), apple (Wang *et al*., 2007; Li *et al*., 2016), and citrus fruit (Yin *et al*., 2016). However, ERFs have been linked with plant environmental responses, particularly to low temperature stress (Yin *et al*., 2012; Zeng *et al*., 2015; Wang *et al*., 2017). In fact, the C-repeat/DRE-Binding Factor (CBF) are members of the ERF family that are crucial for cold acclimation in plants (Fowler and Thomashow, 2002; Thomashow, 2010; Barrero-Gil *et al*., 2016).

1.3.4. Other ripening-related TFs

Apart from the TF family mentioned above, there are several other minor TFs that have been shown to modulate specific fruit ripening attributes. MYB TFs play an important role in flavonoid biosynthesis pathway, and in tomato, MYB10 was shown to regulate anthocyanin levels during fruit ripening (Adato et al., 2009; Ballester et al., 2010). Different MYBs have also been linked with anthocyanin biosynthesis during fruit ripening, strawberry (Schaart *et al*., 2013), peach (Rahim *et al*., 2014); grapevine (Czemmel *et al*., 2012), loquat (Zeng *et al*., 2015), and apple (Takos *et al*., 2006; Espley *et al*., 2007). Recently, R2R3-MYB transcription factor has been shown to regulate chlorophyll degradation and carotenoid accumulation in kiwifruit (Ampomah-Dwamena *et al*., 2019).

Homeobox proteins have also been associated with fruit ripening regulation. In tomato, Lin *et al*. (2008) identified a putative HD-zip homeobox protein (HB1) that binds to the promoters of *ACO* genes. Most recently, four HD-zip TFs including MaHDZI.19, MaHDZI.26, MaHDZII.4 and MaHDZII.7 have been shown to promote fruit ripening by activating ethylene biosynthetic and cell wall modifying genes in banana (Yang *et al*., 2019). Basic leucine zippers have been shown to regulate fruit ripening in banana (Hu *et al*., 2016), peach (Lovisetto *et al*., 2013), and grape (Nicolas *et al*., 2014). In several fruit, basic helix-loop-helix (bHLH) TFs have also been linked to anthocyanin accumulation during ripening (Tani *et al*., 2011; Xie *et al*., 2012; Sun *et al*., 2015). Other TFs that have been shown to play a crucial role during fruit ripening include WRKY (Cheng *et al*., 2016), zinc finger (Weng *et al*., 2015; Han *et al*., 2016), TCP (Guo *et al*., 2018; Song *et al*., 2018), and GRAS (Huang *et al*., 2015; Zhang *et al*., 2018).

1.4. Other phytohormones that regulate fruit ripening

Besides ethylene, other hormones such as auxin, abscisic acid, jasmonate and gibberellin have also been linked to fruit ripening regulation (McAtee *et al*., 2013; Kumar *et al*., 2014). These hormones achieve this by acting either in conjunction with ethylene or independently of ethylene.

1.4.1. Auxin

Indole-3-acetic acid (IAA), the major auxin in plants, was shown to be involved in a crosstalk with ethylene to regulate fruit ripening in peach (Trainotti *et al*., 2007; Tatsuki *et al*., 2013). In tomato, several auxin signalling-related TFs, the auxin responsive factors (ARF), were shown to regulate tomato fruit ripening (Sagar *et al*., 2013; Hao *et al*., 2015). Cruz *et al*. (2018) demonstrated that the interaction between light, ethylene and auxin was involved in the regulation of carotenoid metabolism in tomato.

1.4.2. Abscisic acid (ABA)

ABA seems to have a stronger fruit ripening regulatory role in non-climacteric fruit (McAtee *et al*., 2013). However, most fleshy fruit exhibit an increase in ABA content during fruit ripening and any treatment that delays this increase has been found to delay the fruit ripening process as well (Zhang *et al*., 2009). In tomato, Sun *et al*. (2012) demonstrated that ABA promotes fruit ripening by enhancing ethylene biosynthesis via upregulation of ethylene biosynthetic genes. ABA has also been shown to modulate fruit ripening in citrus by promoting carotenoid biosynthesis (Rodrigo *et al*., 2003).

1.4.3. Gibberellin (GA)

The role of GA in fruit ripening has been extensively reviewed in citrus fruit whereby application of GA was shown to delay fruit ripening (Alós *et al*., 2006; Rodrigo and Zacarias, 2007; Rios *et al*., 2010). Yamaguchi (2008) argued that the rate of GA biosynthesis and deactivation is the criteria for determining GA bioactivity. In this regard, kiwifruit ripening was shown to be accompanied by an increase in the expression of a gene encoding gibberellic acid-2-oxidase (GA2ox), which is associated with GA degradation (Atkinson *et al*., 2011; Asiche *et al*., 2018).

1.4.4. Jasmonates

Jasmonates including jasmonic acid (JA) and methyl jasmonate (MeJA) are important regulators of plant responses to abiotic and biotic stresses (Wasternack and Hause, 2013). However, JAs have also been found to play a role in fruit ripening regulation. JAs stimulate ethylene biosynthesis in climacteric fruit such as pear and tomato, resulting in accelerated fruit ripening (Liu *et al*., 2012; Concha *et al*., 2013; Zhang *et al*., 2014). In apple, the JA-activated TF MdMYC2 was shown to regulate fruit ripening via interaction with ERF and ethylene biosynthetic genes (Li *et al*., 2017).

1.5. Low temperature and fruit ripening regulation

Temperature is the most important environmental factor that influences the postharvest life of fruit and other fresh horticultural produce. This is due to the ability of temperature to influence the both internal and external factors affecting fruit quality such as general metabolic and developmental changes, water loss, physiological disorders and pathogen attack (McGlasson *et al*., 1979; Hardenburg *et al*., 1986).

1.5.1. Low temperature as a postharvest technology

Low temperature is a major postharvest technology that is used widely to extend the postharvest life of fruit and vegetables. Indeed, most postharvest protocols recommend a cold chain network right from harvest to the point of purchase by the consumer in order to minimize the incidence of postharvest losses (McGlasson *et al*., 1979; Gross *et al*., 2016). The popularity of cold storage in postharvest handling is founded on the ability of low temperature to slow most cell metabolic activities including those related to fruit ripening and plant senescence. In addition, low temperature inhibition of fruit ripening is attributed to a delay in the onset of system II ethylene production (Gross *et al*., 2016). Low temperature also retards the growth of most postharvest fruit pathogens, which alleviates the deteriorative effects of disease-induced ethylene (Everett, 2003; Wang and Wang, 2009; Vico *et al*., 2011; Manning *et al*., 2016). However, fruit quality deterioration is known to occur at every point in the postharvest cycle and therefore, low temperature storage is often integrated with other approaches such as ethylene removal, controlled and modified atmosphere, and use of ethylene antagonists.

1.5.2. Chilling injury in fruit

Despite the wide use of low temperature storage to extend the postharvest life of horticultural produce, certain tropical and subtropical fruit are not suitable for this kind of storage. In these chilling-sensitive fruit, suboptimal low storage temperatures trigger the developmental of physiological disorders termed chilling injury that negatively affect fruit quality (Lyons, 1973; Sevillano *et al*., 2009). The most common chilling injury symptoms include tissue browning, woolly or dry texture, and abnormal cell metabolism such as membrane permeability disorders (Sevillano *et al*., 2009). Chilling injury symptoms activate ethylene biosynthesis and signalling elements (Wang *et al*., 2009), and this further aggravates the deteriorative processes during storage.

The sensitivity to low temperature and the nature of chilling injury symptoms differs depending on the fruit species and cultivar. Banana fruit are usually considered very sensitive to chilling as chilling symptoms including peel darkening and browning appear at relatively high storage temperatures (12 ºC) (Nguyen *et al*., 2003). In kiwifruit, the recommended storage temperatures for long-term storage are 0–4 ºC (Arpaia *et al*., 1987; Pranamornkith *et al*., 2012). Extended storage at temperatures below this recommended range result in the development of chilling injury symptoms manifested as low temperature breakdown (Lallu, 1997; Jabbar and East, 2016), and lignification (Li *et al*., 2017; Suo *et al*., 2018). Exposure of citrus fruit to low temperatures induces chilling injury symptoms that can include pitting, necrosis, and staining in the flavedo tissue (Lafuente *et al*., 2001). Chilling injury symptoms in tomato fruit occur after extended storage at below 8 ºC and include surface pitting, flavour loss and blotchy ripening among others (Wang, 1993; Zhang *et al*., 2016). Therefore, proper management of storage temperature is required to balance between the preservation of fruit quality and prevention of chilling injury symptom appearance.

1.5.3. Promotion of fruit ripening by low temperature

1.5.3.1. Kiwifruit

Kiwifruit are classified as climacteric fruit as most of the ripening-associated changes including softening, sugar increase, acidity reduction and aroma volatile production are triggered by ethylene (Pratt and Reid, 1974). However, kiwifruit exhibit a unique ripening pattern as system II (autocatalytic) ethylene production occurs when the fruit have already ripened to eating

quality (Richardson *et al*., 2011). The role of ethylene in on-vine fruit ripening therefore remains unclear.

During low temperature storage, kiwifruit are known to soften extensively in the absence of any detectable increase in ethylene production (Kim *et al*., 1999; Antunes, 2007; Yin *et al*., 2009). Because of limited experimental evidence, this unique ripening behaviour exhibited by kiwifruit has been attributed to the basal levels of system I ethylene, as it was demonstrated that kiwifruit can be induced by extremely low ethylene concentrations (Pranamornkith *et al*., 2012; Jabbar and East, 2016). To support this hypothesis, it has been suggested that the ripening is caused by a synergy between ethylene and low temperature, that is, low temperature storage increases the sensitivity of kiwifruit to trace levels of ethylene (Murakami *et al*., 2014; Minas *et al*., 2016). However, this hypothesis is limited in the fact that no comparisons were made to the ripening pattern during room temperature storage.

Storage of kiwifruit at room temperature is an uphill task since kiwifruit are known to be susceptible to pathogen attack especially *Botryoshaeria* spp., *Phomopsis* spp., and *Diaporthe actinidia* (Yano and Hasegawa, 1993; Kinugawa, 2000; Koh *et al*., 2005; Manning *et al*., 2016). Disease-infected fruit produce stress ethylene that can trigger ethylene-dependent ripening in adjacent fruit. Therefore, careful handling to eliminate the effects of disease-induced ethylene is crucial in studies of the mechanism of fruit ripening in kiwifruit under either low or room temperature conditions.

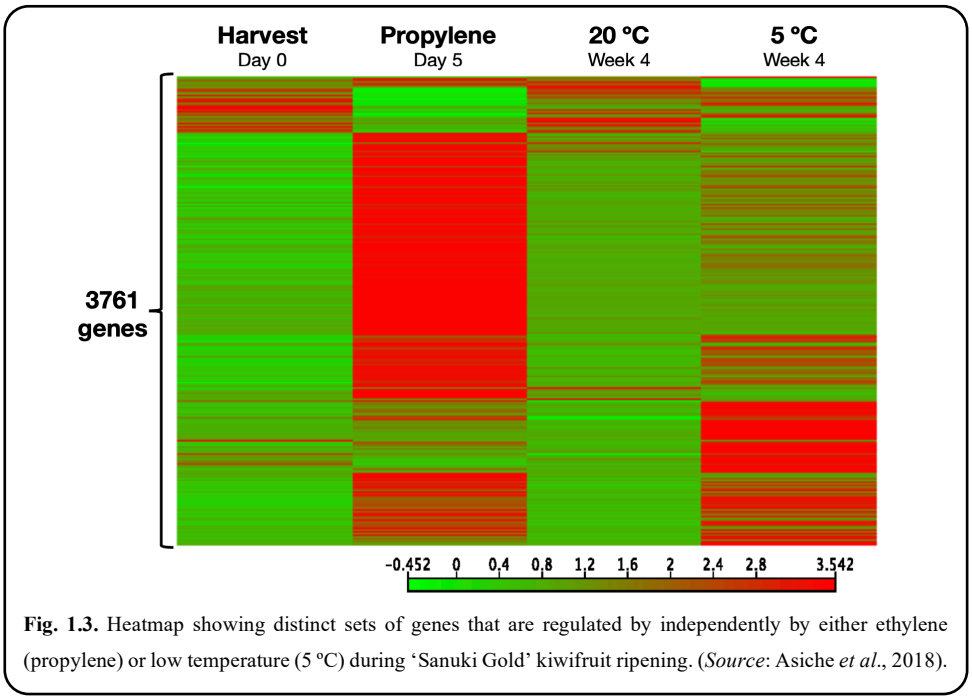


Fig. 1.3. Heatmap showing distinct sets of genes that are regulated by independently by either ethylene (propylene) or low temperature (5 °C) during 'Sanuki Gold' kiwifruit ripening. (*Source*: Asiche *et al*., 2018).

In an attempt to understand the mechanisms involved in kiwifruit ripening during low temperature storage, my predecessors demonstrated that kiwifruit ripening occurred faster during storage at 5 ºC than at 20 ºC in the absence of any measurable increase in ethylene production (Mworia *et al*., 2012; Asiche *et al*., 2017; Asiche *et al*., 2018). Furthermore, blocking ethylene signalling by repeated 1-MCP treatments failed to inhibit the accelerated fruit ripening during low temperature storage. These observations indicated that kiwifruit ripening was inducible by low temperature independently of ethylene. As a follow-up to this conclusion, Asiche *et al*. (2018) demonstrated by RNA-seq analysis that low temperature regulated a distinct ripening-related set of genes in kiwifruit independently of ethylene (Fig. 1.3). Nevertheless, research on low temperature regulation of fruit ripening in kiwifruit remains underexplored.

1.5.3.2. European pears

The ripening of European pear fruit relies on the initiation of autocatalytic ethylene production which triggers dramatic flesh softening, aroma volatile production and in some cultivars, skin colour change (Lelièvre *et al*., 1997b; Itai *et al*., 1999; Hiwasa *et al*., 2003). European pear fruit ripening can also be advanced by treatment with ethylene or its analogue propylene (Hiwasa *et al*., 2003). Most European pear cultivars attain optimum soluble sugar content and acidity level at the commercial maturity stage and thus, ethylene does not have a significant influence on their content.

European pears are unique in that most of the cultivars exhibit some resistance to ripening even at the correct maturity. Therefore, the common practice is to store the fruit at low temperature conditions for a specified period of time to trigger fruit ripening (Villalobolos-Acuna and Mitcham, 2008). Early-maturing cultivars such as ‘Bartlett’ and ‘La France’ require a relatively shorter chilling period (10–21 d) to induce ripening (Hiwasa *et al*., 2003; Nham *et al*., 2017), whereas late-maturing cultivars like ‘Passe Crassane’ have a long chilling requirement of up to 3 months (Lelievre *et al*. 1997b; El-Sharkawy *et al*., 2003). ‘Passe Crassane’ fruit are inexplicable since they fail to produce autocatalytic ethylene and ripen during storage at room temperature even for 145 d (El-Sharkawy *et al*., 2004).

Many studies have demonstrated that low temperature can activate the expression of ethylene biosynthetic genes, *ACSs* and *ACOs*, which enables the fruit to produce autocatalytic system II

ethylene that is required for fruit ripening (Lelievre *et al*. 1997b; El-Sharkawy *et al*., 2003; Hiwasa *et al*., 2003; Villalobolos-Acuna and Mitcham, 2008). Upon low temperature storage, increased ethylene production triggers the expression of various softening related genes (Fonseca *et al*., 2005). However, the physiological and molecular changes triggered by low temperature resulting in increased ethylene production and fruit ripening remain to be seen.

1.5.3.3. Citrus fruit

Unlike kiwifruit and European pears, citrus fruit are generally classified as non-climacteric since mature fruit produce trace levels of ethylene (system I) and ethylene is not required for normal ripening (Eaks, 1970; Sawamura, 1981; Katz *et al*., 2004). However, treatments with ethylene have been shown to advance the peel degreening process (Purvis and Barmore, 1981), forming the basis for the wide use of this hormone for commercial degreening purposes (Porat, 2008; Mayuoni *et al*., 2011). Nevertheless, the primary regulator of natural peel degreening which occurs in the presence of trace levels of system I ethylene remains unknown.

The progression of on-tree peel degreening in various citrus cultivars requires extended cold periods below 13 ºC (Manera *et al*., 2012; Manera *et al*., 2013; Rodrigo *et al*., 2013; Conesa *et al*., 2019). Peel degreening is also accelerated by low/intermediate storage temperatures (6–15 ºC) (Matsumoto *et al*., 2009; Van Wyk *et al*., 2009; Zhu *et al*., 2011; Carmona *et al*., 2012a; Tao *et al*., 2012). The promotion of peel degreening by low temperature is attributed to the stimulation of chlorophyll degradation and carotenoid accumulation (Matsumoto *et al*., 2009; Carmona *et al*., 2012a; Tao *et al*., 2012). Like in kiwifruit, low temperature promotion of citrus peel degreening is also attributed to trace levels of system I ethylene that are present in mature fruit (Goldschmidt *et al*., 1993; Carmona *et al*., 2012b). The underlying regulatory mechanisms involved in natural peel degreening have therefore remained a mystery.

1.6. Project goal and objectives

A majority of fruit ripening studies have, up to this point, focussed on the role of ethylene. As a result, accelerated fruit ripening observed during low temperature storage is often attributed to ethylene signalling even though there are no direct evidences. The current understanding of the molecular mechanisms underlying low temperature regulation of fruit ripening are thus limited. The aim of this project was to address this issue by carrying out comprehensive physiological and transcriptome analyses to compare ethylene-dependent and low temperature-

modulated fruit ripening changes in various cultivars of kiwifruit, citrus and European pear. To achieve this, the role of low temperature in fruit ripening regulation is discussed in this thesis based on the following specific objectives:

i. To determine the sufficient low temperature that is required to induce fruit ripening in the absence of ethylene.
ii. To examine cultivar differences in the responses of kiwifruit to low temperature-modulated fruit ripening.
iii. To investigate the role of low temperature in the regulation of on-vine and on-tree fruit ripening.
iv. To determine the optimum temperature for long-term storage of kiwifruit which can counteract premature ripening by low temperature.
v. To evaluate the differences in quality attributes between fruit ripened by ethylene and low temperature.
vi. To find out the physiological and transcriptional changes involved in low temperature stimulation of fruit ripening in 'Passe Crassane' pear fruit.
vii. To establish the role of low temperature in regulation of peel degreening and associated gene expression in lemons and satsuma mandarins.

CHAPTER 2

Low temperature regulation of fruit ripening in kiwifruit

2.1. Low temperature regulates fruit ripening in kiwifruit via a dose × time-dependent mechanism

Abstract

Kiwifruit ripening has been shown to be modulated by low temperature independently of ethylene. However, the adequate low temperature that can sufficiently induce fruit ripening remains unclear. Changes in fruit ripening and associated gene expression in 'Rainbow Red' kiwifruit were compared both during storage at different temperatures and on-vine fruit ripening. Storage at 5 ºC and 10 ºC stimulated fruit ripening to eating quality (~6 N) within four weeks, which was accompanied by increased expression levels of *AcACO3*, *AcXET2*, *AcEXP1*, *AcPG*, *AcPMEi*, *AcSUS*, *Acβ-AMY1*, *Acβ-AMY2*, *AcGA2ox2*, *AcNAC3*, *AcNAC4*, and *AcMADS2*. Fruit stored at 15 ºC required a relatively longer exposure time (eight weeks) to attain eating quality, and this concurred with a delayed accumulation of fruit ripening-related mRNAs. Accelerated fruit ripening at 5 ºC, 10 ºC and 15 ºC was not inhibited by repeated 1-MCP treatments, indicating that it was independent of ethylene. On the other hand, fruit at 22 ºC exhibited the slowest ripening rate and the expression of ripening-related genes was maintained at minimal levels. On-vine fruit ripening rate was initially slow at the early stages when the average field temperature was ~20 °C, but it occurred rapidly with increased expression of ripening-related genes when the environmental temperatures dropped to ≤ 15 ºC. These results demonstrated that kiwifruit ripening both on the vines and during storage was transcriptionally modulated by low temperature independently of ethylene.

2.1.1. Introduction

Kiwifruit ripening is a complex process that includes flesh softening, a reduction in titratable acidity (TA), an increase in soluble solids content (SSC), and production of aroma volatiles (Pratt and Reid, 1974). These ripening-associated changes can be advanced by treatment with exogenous ethylene or propylene (Antunes *et al*., 2000; Mworia *et al*., 2010), and hence the classification of kiwifruit as climacteric. However, kiwifruit are also known to extensively ripen during low temperature storage in the absence of any detectable ethylene increase (Kim *et al*., 1999; Antunes, 2007).

The unique fruit ripening behaviour exhibited by kiwifruit has often been attributed to ethylene signalling, that is, basal levels of system I ethylene are physiologically active (Kim *et al*., 1999). This argument is supported by the observations that extremely low ethylene levels (0.01 μLL^{-1}) can trigger kiwifruit ripening during low temperature storage (Pranamornkith *et al*., 2012; Jabbar and East, 2016). Low temperature was also reported to increase the sensitivity of kiwifruit to ethylene (Murakami *et al*., 2014; Minas *et al*., 2016). However, the major shortcoming of previous studies is that no comparisons were made to fruit stored at room temperature. In addition, there were no attempts in previous studies to eliminate the effects of disease-induced ethylene, which is known to cause a havoc during postharvest storage of kiwifruit (Yano and Hasegawa, 1993; Kinugawa, 2000; Koh *et al*., 2005; Manning *et al*., 2016).

Recent studies have demonstrated that kiwifruit ripening is inducible by low temperature independently of ethylene. In the absence of ethylene, kiwifruit ripening occurred faster, and the expression levels of associated genes were higher during storage at 5 ºC than at 20 ºC (Mworia *et al*., 2012; Asiche *et al*., 2017; Asiche *et al*., 2018). A follow-up study by Asiche *et al.* (2018) further revealed that a distinct set of fruit ripening-associated genes were induced in 'Sanuki Gold' kiwifruit during storage at 5 ºC, but not at 20 ºC. However, it remains unclear how other storage temperatures can affect fruit ripening and associated gene expression in kiwifruit.

'Rainbow Red' kiwifruit are an important early-maturing cultivar in Japan having a high SSC (> 18 %) and low TA (~1 %) (Murakami *et al*., 2014; Asiche *et al*., 2017). On-vine ripening of 'Rainbow Red' fruit has been reported to proceed in the absence of any detectable increase in ethylene production (Murakami *et al*., 2015). In 'Sanuki Gold' kiwifruit, it was demonstrated that on-vine fruit ripening and associated gene expression showed strong similarities to low temperature-induced ripening patterns. Research on this phenomenon is however limited and therefore, it is remains unclear whether low temperature response is involved in regulation of on-vine fruit ripening.

In this study, the main goal was to determine the effect of different storage temperatures on fruit ripening and related gene expression and to establish the role of low temperature in the regulation of on-vine ripening in 'Rainbow Red' kiwifruit.

2.1.2. Materials and methods

2.1.2.1. Plant materials and treatments

'Rainbow Red' kiwifruit (*Actinidia chinensis*) were obtained from a commercial orchard in Takamatsu, Japan. Ethylene-induced ripening was conducted as previously reported (Asiche *et al*., 2018). Commercially mature fruit were harvested on 11th September 2012 and treated continuously with 5000 μLL^{-1} of propylene at 20 °C in a container fitted with soda lime to absorb the carbon dioxide produced by respiration. Non-treated fruit were also held in air at 20 °C as a control.

For storage tests, healthy fruit harvested on 19th September 2014 were divided into four groups for storage at either 5 °C, 10 °C, 15 °C, or 22 °C for eight weeks using the individual separation technique that was previously described by Asiche *et al*. (2018). Each storage temperature had a corresponding group of fruit that were repeatedly treated with 1-MCP (SmartFresh™; Rohm and Hass, Philadelphia, PA, USA). 1-MCP treatment (5 μLL^{-1} for 12 h) was carried out twice according to the procedure described by Mworia *et al*. (2012). To monitor for quiescent disease infections, ethylene production of all fruit was measured individually throughout the storage period. Fruit that started to produce ethylene were set aside and monitored in a separate room. These fruit developed ripening rot symptoms within a few days indicating that the detected ethylene was disease-induced. Healthy fruit with no detectable ethylene production were sampled at harvest, and after four and eight weeks of storage.

On-vine sampling was from eight harvests: 19th September, 2nd October, 16th October, 27th October, 4th November, 25th November, 5th December and 17th December. Changes in environmental temperature were also monitored during the on-vine ripening period.

2.1.2.2. Measurement of ethylene production

Ethylene measurement was conducted using a gas chromatograph (model GC-8A; Shimadzu, Kyoto, Japan), equipped with a flame ionization detector (set at 200 °C) and an activated alumina column (set at 80 °C), as previously described by Mworia *et al*. (2010).

2.1.2.3. Evaluation of fruit quality parameters

Fruit firmness was measured using a penetrometer (model SMT-T-50, Toyo Baldwin, Tokyo, Japan) fitted with a 5 mm plunger at a speed of 30 mm min^{-1}). SSC was measured from juice extracts using a digital refractometer (Atago Co. Ltd, Tokyo, Japan) and expressed as a percentage. TA was determined by titrating the juice extract against 0.1 N NaOH using phenolphthalein as the indicator and expressed as percentage citric acid equivalents. Each sampling point consisted of five fruit. The outer pericarp of three independent biological replicates for each sampling point was cut into pieces, frozen in liquid nitrogen, and stored at -80 °C for further analysis.

2.1.2.4. RNA extraction, cDNA synthesis, and Real-Time PCR

Total RNA was extracted from three biological replicates using a method for polysaccharide-rich fruit (Ikoma *et al*., 1996) with slight modifications. After RNA extraction, DNase I treatment and RNA clean-up were done using FavorPrep After Tri-Reagent RNA Clean-up kit (Favorgen Biotech co., Ping-Tung, Taiwan). First strand cDNA was synthesized from 2.4 µg DNase I treated RNA using RevTra Ace reverse transcriptase (Toyobo, Osaka, Japan) and random hexamer primer according to the manufacturer's instructions. Table 2.1 indicates the gene accessions and primers used for RT-qPCR analysis in this study. Gene expression was calculated by $2^{-\Delta Ct}$ and normalized using actin as the housekeeping gene. Mature fruit sampled at harvest were set as calibrators for calculating relative expression levels.

Table 2.1. Kiwifruit primers used for Real-Time PCR analysis. Gene sequences were obtained from the Kiwifruit Genome Database and NCBI Database and primers designed using Primer3 software (version 0.4.0, http://bioinfo.ut.ee/primer3-0.4.0/)

Gene	Access. No.	*Name*	Forward (5' to 3')	Reverse (5' to 3')
AcACS1	Achn364251	*ACC synthase*	GAAAGGCTGCGTGCAATTCTC	CCTGAAAATGGACTGCCCATC
AcACO2	Achn326461	*ACC oxidase 2*	TCTCAGAAATCCCCGATCTCG	TTGGAGCCACTGAAAGCCTTC
AcACO3	Achn150611	*ACC oxidase 3*	CAGATGGCAACAGAATGTCG	AACTTGAGGCCAGCATAGAG
AcPL2	HQ108112	*Pectate lyase 2*	AAGACGAGCCACTATGGATC	CTGGCTTGCAATCGTGTATG
AcPG	AF152756	*Polygalacturonase*	TGGATTTGTTAGGGGTGTGC	CAACTTGTGTCGCTGATGAC
AcEXP1	AY390358	*Expansin 1*	CGTGCTTCGAGCTAAAGTGC	CGGCGATCTTGAGGAACATG
AcXET1	Achn349851	*Xyloglucan endotransglycosylase 1*	CGTTCGATCAACCGATGAAGA	TCTTGTAAGCAGCCACGAAGG
AcXET2	Achn387971	*Xyloglucan endotransglycosylase 2*	GCTTCTCACGCTTTCTCTCG	GTTGACCCCAACGAAGACAG
AcPMEi	FG458520	*Pectin methyl esterase inhibitor*	CTTTGCATCACCTCGCTCTC	ACTGGGACAGCTTCACCATC
AcßAMY1	FG525163	*ß-Amylase 1*	CCCCACATTGATGGAATGAC	GTTTGTGATGCTGCCACTCG
AcßAMY2	Achn212571	*ß-Amylase 2*	CAGAGAACGCAAACTGCTCG	GTTCCCGGAGTCTGATCTAC
AcSUS	FG439911	*Sucrose synthase*	TGCCGAATTACAGGGTGTTC	CTCCAAGGCATGAGCAATGG
AcGA2ox2	Achn218871	*Gibberellic acid oxidase 2*	CCTGCTCATGAACTTCCTCG	ATTCGTCGTAGGGTTTGGTG
AcMADS2	Achn235371	*MADS transcription factor*	GGACAAGAACAGTCGCCAGG	GTATCTGTCGCCGGTGATG
AcERF6	GQ869857	*Ethylene response factor 6*	ACGGCATCGAAAACCGTTC	TGCCGGATTCCTCTGTACTTG
AcNAC3	Achn289291	*NAC transcription factor 3*	CGCTTATGTCCCTTCCATGTC	CAAAGCCCTGAGTGAATCCAG
AcNAC4	Achn169421	*NAC transcription factor 4*	GAATTGCCCGAGAAAGCAGA	TGTCTTGATACCCTTCGGTGG
AcACTIN	EF063572	*Actin*	TGGAATGGAAGCTGCAGGA	CACCACTGAGCACAATGTTGC

2.1.3. Results

2.1.3.1. Ethylene-induced kiwifruit ripening

The determination of ethylene-induced ripening in 'Rainbow Red' kiwifruit was carried out by Asiche *et al*. (2018). Continuous propylene treatment triggered ethylene production, fruit softening, SSC increase and TA reduction within 5 d (Fig. 2.1.1). Non-treated fruit showed no significant changes in the above ripening-associated changes after 7 d.

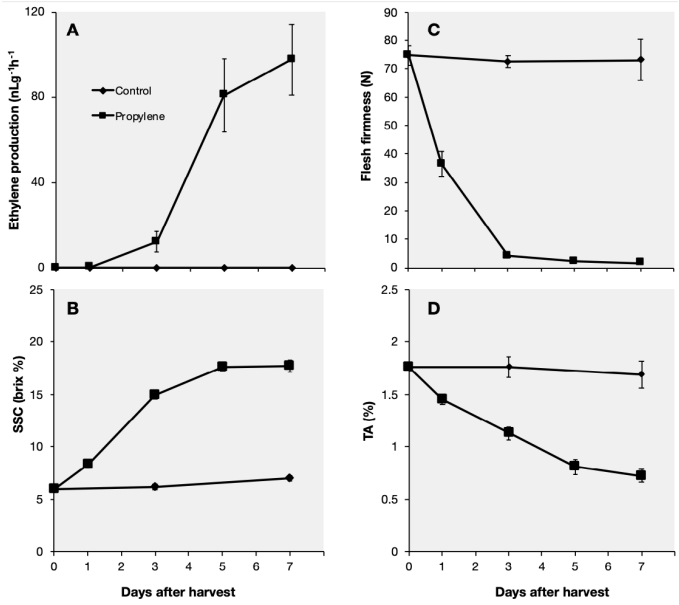


Fig. 2.1.1. Ethylene production (A), outer pericarp firmness (C), soluble solids concentration (B) and titratable acidity (D) of control and propylene treated 'Rainbow Red' kiwifruit. Propylene treatment was done continuously at a concentration of 5000 µL L^{-1} at 20 °C. Data points are means and vertical bars represent SD (n=5). (*Source: Asiche et al., 2018*).

2.1.3.2. Kiwifruit ripening behaviour at different storage temperatures

Healthy 'Rainbow Red' kiwifruit that did not produce any detectable ethylene exhibited ripening responses (at least to some extent) at all storage temperatures (Fig. 2.1.2). Reduction in fruit firmness occurred fastest at 5 ºC and 10 ºC, from 58 N at harvest to 5–10 N after 4 weeks and 2 N after 8 weeks (Fig. 2.1.2A). Fruit firmness also decreased at a moderate rate during storage at 15 ºC, reaching 27 N and 7 N after 4 weeks and 8 weeks, respectively. However, softening occurred very slowly at 22 ºC with fruit registering a high firmness (33 N) after 8 weeks. Fruit TA decreased from 1.75 % at harvest to ~0.8 % after 8 weeks of storage at 5 ºC and 10 ºC, and 1.1 % at 15 ºC at the same timepoint (Fig. 2.1.2C). No significant changes in TA were observed in fruit at 22 ºC with 1.58 % after 8 weeks. The SSC increased during storage at all temperatures although the levels were generally higher in fruit at 10 ºC at the end of the storage period (Fig. 2.1.2B).

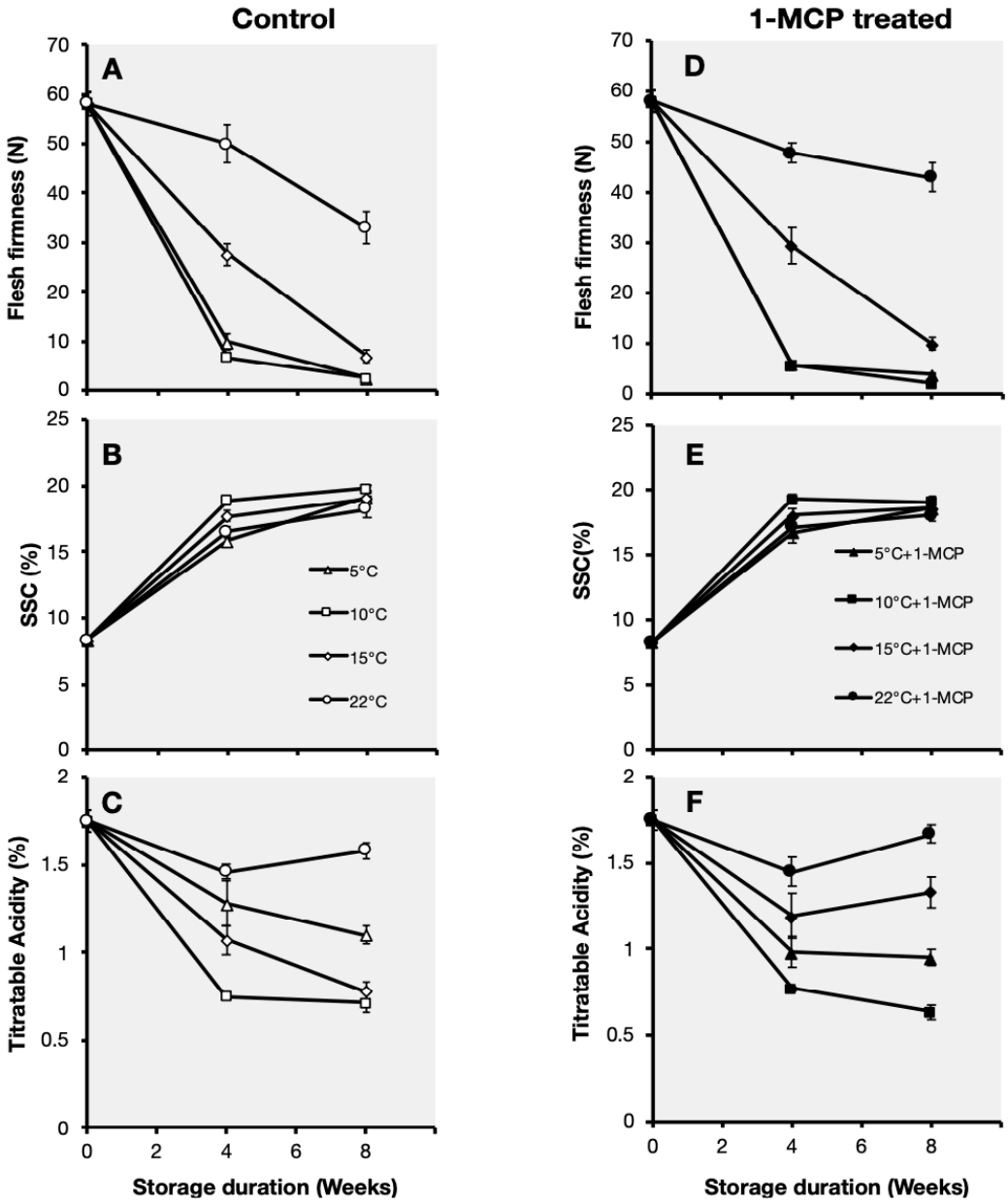


Fig. 2.1.2. Effect of storage temperature on ripening characteristics of control (A, B, C) and 1-MCP treated (D, E, F) 'Rainbow Red' kiwifruit. Fruit with undetectable ethylene production were sampled. Data points represent means and vertical bars represent standard deviations (n = 5).

2.1.3.3. On-vine kiwifruit ripening behaviour

While no detectable ethylene production was detected, undetached lemon 'Rainbow Red' fruit gradually lost their firmness, increased their SSC and decreased their TA from 19th September to 17th December (Fig. 2.1.3). Both firmness and TA reduction occurred slowly between 19th September and 16th October when the minimum environmental temperatures were 15–20 ºC, but the changes were rapid between 16th October and 25th November when the minimum temperatures were ≤ 10 ºC. Fruit SSC increased steadily throughout the on-vine ripening period.

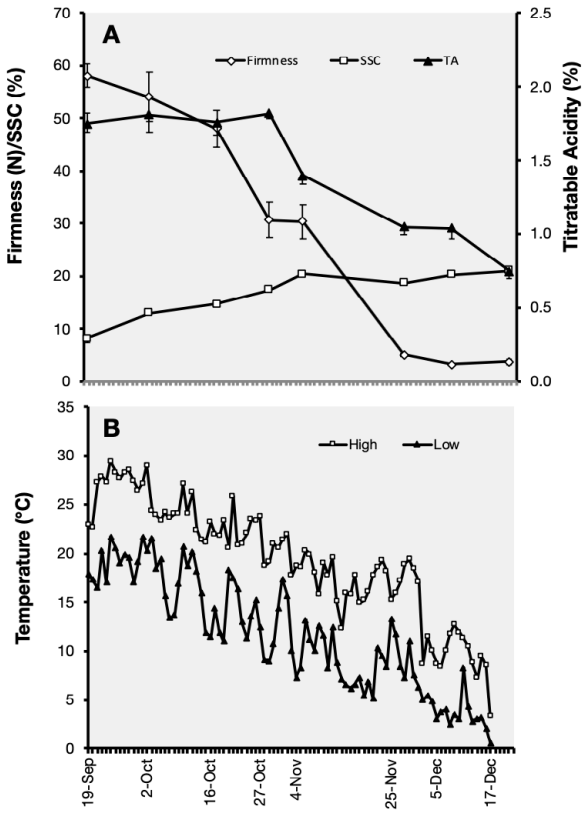


Fig. 2.1.3. (A) Fruit ripening characteristics of 'Rainbow Red' kiwifruit attached to the vines. (B) Changes in environmental temperature during the experimental period. Fruit with undetectable ethylene production were sampled. Data points represent means and vertical bars represent standard deviations (n = 5).

2.1.3.4. Expression of ethylene biosynthetic genes

Propylene treatment for 5 d dramatically increased the expression levels of *AcACS1* (9500-fold) and *AcACO2* (5500-fold) in 'Rainbow Red' kiwifruit (Fig. 2.1.4A, B). Both *AcACS1* and *AcACO2* showed minimal expression changes during storage at all temperatures, as well as during on-vine fruit ripening. By contrast, *AcACO3* showed no expression changes in response to propylene treatment, while its transcript levels notably increased during storage at temperatures lower than 25 ºC as well as during on-vine fruit ripening (Fig. 2.1.4C). Particularly, *AcACO3* expression increased faster within 4 weeks during storage at 5 ºC and 10 ºC than at 15 ºC where it increased only after 8 weeks. No changes in *AcACO3* expression were recorded during storage at 22 ºC.

2.1.3.5. Expression of cell wall-modifying genes

The expression of *AcXET1* and *AcPL2* showed a dramatic increase in response to propylene treatment for 5 d, whereas no changes were registered during storage and on-vine fruit ripening (Fig. 2.1.5A, C). Conversely, *AcXET2* expression showed no changes in response to propylene treatment, but the transcripts highly accumulated during storage particularly at 5 ºC (220-fold)

after 8 weeks (Fig. 2.1.5B). It is worth noting that *AcXET2* expression did not change significantly during storage at either 10 ºC, 15 ºC or 22 ºC. During on-vine fruit ripening, *AcXET2* expression showed a relatively slight increase especially on 17th December. *AcEXP1*, *AcPG*, and *AcPMEi* showed a similar expression pattern in that they were upregulated in response to propylene treatment, and during low temperature storage and on-vine fruit ripening (Fig. 2.1.5D–F). The expression levels of *AcEXP1* and *AcPMEi* generally higher during storage at 5 ºC and 10 ºC than 15 ºC; no expression changes were observed in these genes at 22 ºC (Fig. 2.1.5D, F). In addition, *AcPG* expression was substantially higher in fruit at 5 ºC than 10 ºC while no significant changes were recorded at 15 ºC and 22 ºC (Fig. 2.1.5E).

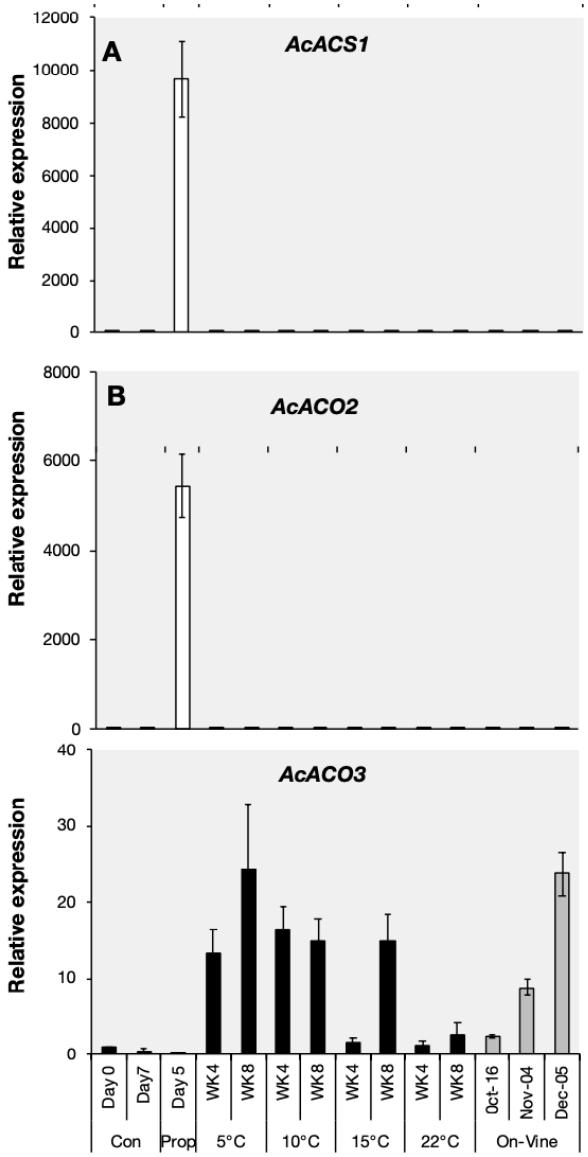


Fig. 2.1.4. Relative expression of ethylene biosynthetic genes in response to propylene treatment, storage temperature and during on-vine ripening in 'Rainbow Red' kiwifruit. Actin was used as the housekeeping gene and Day 0 (harvest) samples were calibrated as 1. Data are the mean ± SE of three fruit (Con – Control, WK – week, Prop – propylene).

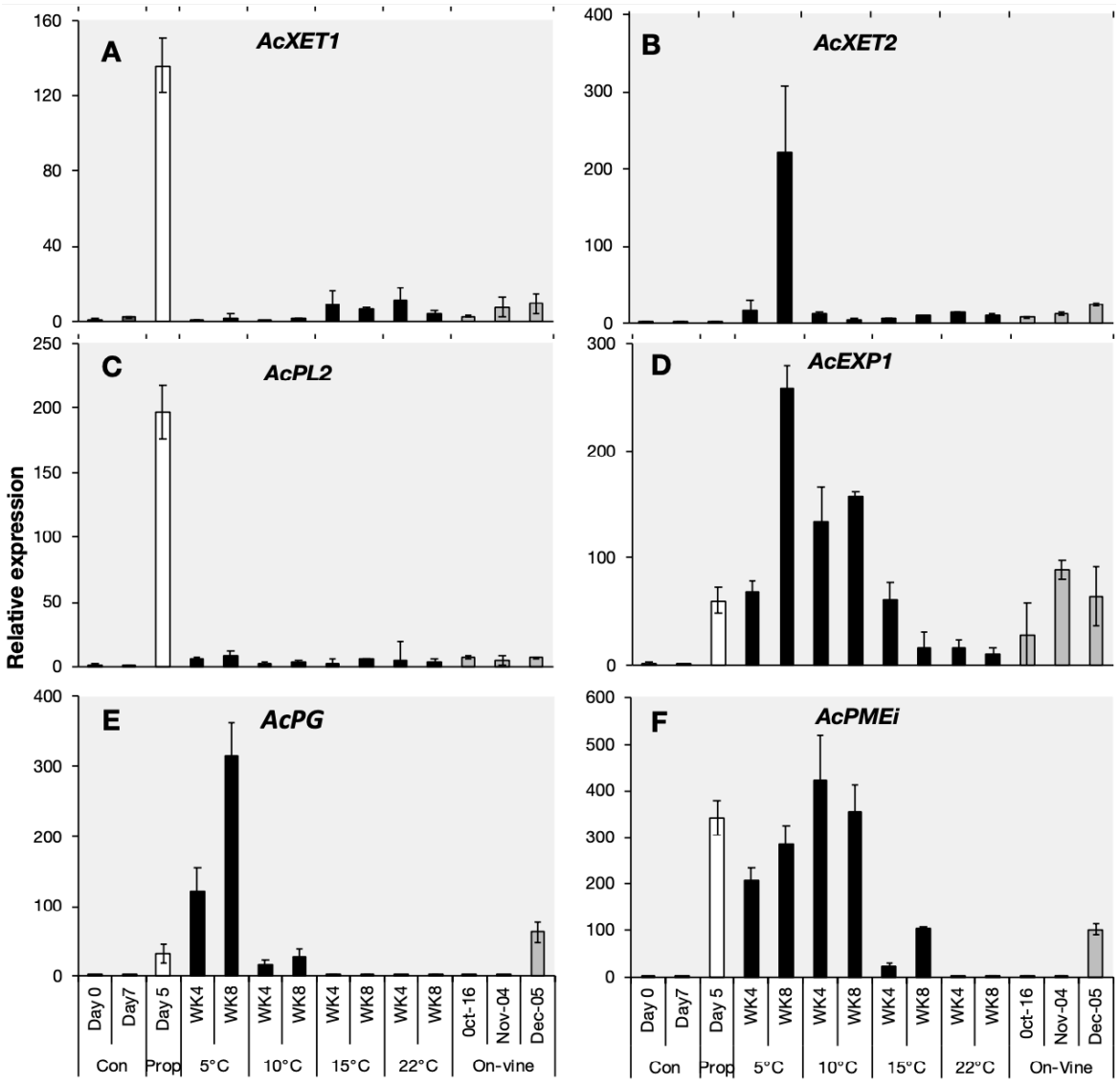


Fig. 2.1.5. Relative expression of cell wall-modifying genes in response to propylene treatment, storage temperature and during on-vine ripening in 'Rainbow Red' kiwifruit. Actin was used as the housekeeping gene and Day 0 (harvest) samples were calibrated as 1. Data is the mean ± SE of three fruit (Con – Control, WK – week, Prop – propylene).

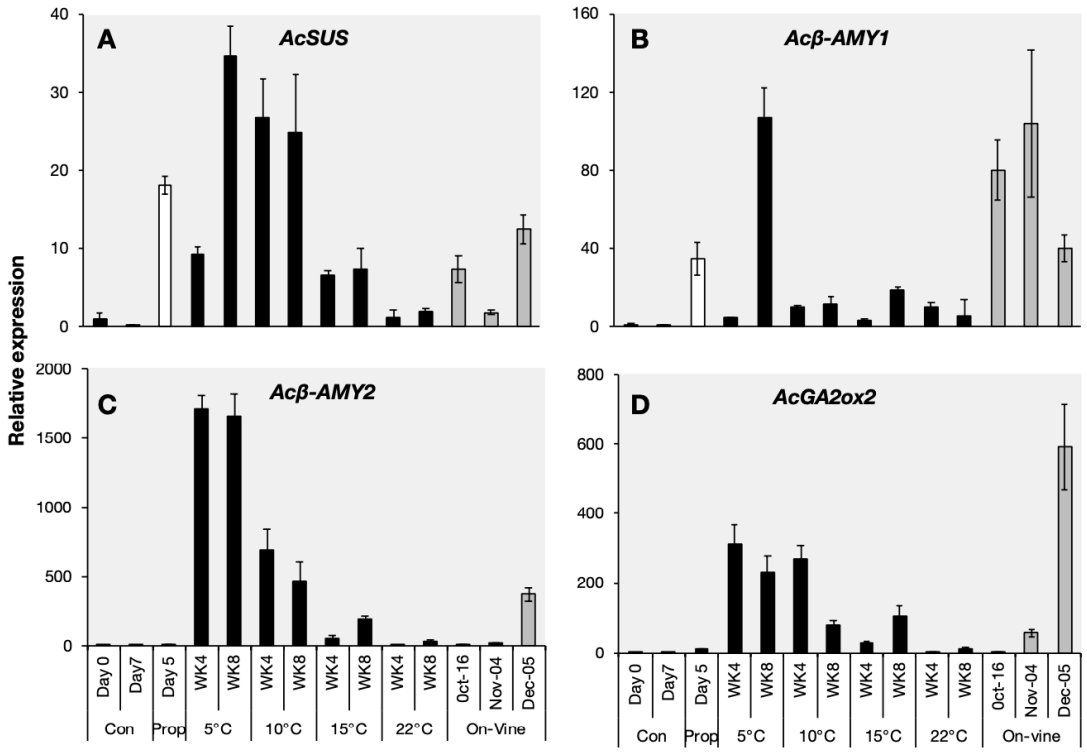


Fig. 2.1.6. Relative expression of carbohydrate metabolism and gibberellins degradation genes in response to propylene treatment, storage temperature and during on-vine ripening in 'Rainbow Red' kiwifruit. Actin was used as the housekeeping gene and Day 0 (harvest) samples were calibrated as 1. Data is the mean ± SE of three fruit (Con – Control, WK – week, Prop – propylene).

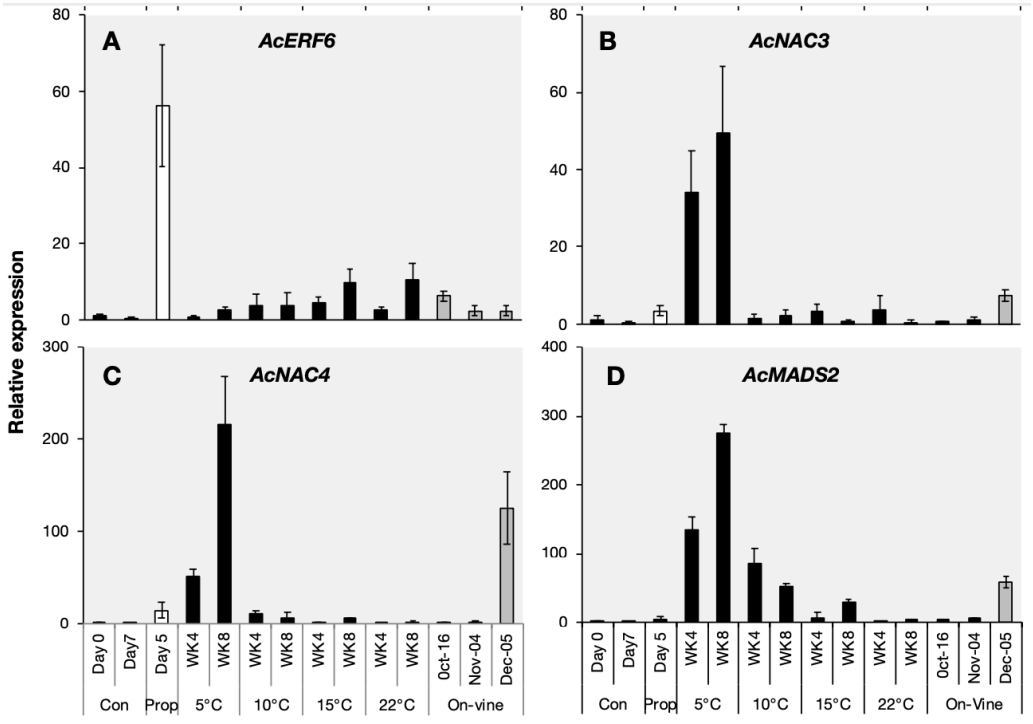


Fig. 2.1.7. Relative expression of ripening-associated transcription factor genes in response to propylene treatment, storage temperature and during on-vine ripening in 'Rainbow Red' kiwifruit. Actin was used as the housekeeping gene and Day 0 (harvest) samples were calibrated as 1. Data is the mean ± SE of three fruit (Con – Control, WK – week, Prop – propylene).

2.1.3.6. Carbohydrate metabolism and gibberellins degradation genes

The expression of *AcSUS* and *Acβ-AMY1* was upregulated in response to propylene treatment, and during storage and on-vine fruit ripening (Fig. 2.1.6A, B). However, both genes showed a higher expression level during storage at 5 ºC and 10 ºC than at 15 ºC with only a slight increase at 22 ºC. On the other hand, the expression of *Acβ-AMY2* and *AcGA2ox2* remained unchanged in propylene-treated fruit, whereas it substantially increased during storage at 5 ºC, 10 ºC and 15 ºC but not at 22 ºC; a substantial increase was also recorded during on-vine fruit ripening (Fig. 2.1.6C, D). It is noteworthy that during storage, the expression levels of both *Acβ-AMY2* and *AcGA2ox2* were appreciably higher at 5 ºC and 10 ºC where they increased after 4 weeks compared to 15 ºC at which they were upregulated only after 8 weeks.

2.1.3.7. Expression of ripening-associated transcription factors

Finally, the expression pattern of selected genes encoding ripening-related TFs was examined. As indicated in Fig. 2.1.7A, *AcERF6* expression increased substantially in response to propylene treatment while only slight changes were observed during storage and on-vine fruit ripening. Transcripts of *AcNAC3* and *AcNAC4* increased slightly in propylene-treated fruit, but the expression levels were significantly higher during storage at 5 ºC and 10 ºC but not at 15 ºC and 22 ºC (Fig. 2.1.7B, C). Both *AcNAC3* and *AcNAC4* were also upregulated during on-vine fruit ripening. Lastly, *AcMADS2* expression did not change in propylene-treated fruit, but it was upregulated during on-vine fruit ripening and storage at 5 ºC, 10 ºC and 15 ºC with little changes at 22 ºC (Fig. 2.1.7D). During storage, *AcMADS2* transcript levels increased more rapidly at 5 ºC and 10 ºC (within 4 weeks) than at 15 ºC (8 weeks).

2.1.4. Discussion

Kiwifruit are generally considered climacteric because exogenous ethylene treatment triggers fruit ripening by regulating the expression of various ripening-associated genes (McMurchie *et al*., 1972; Pratt and Reid, 1974; Antunes *et al*., 2000). This is consistent with the results of this study that propylene treatment induced fruit ripening (Fig. 2.1.1), and upregulated several genes related with ethylene production, softening, carbohydrate metabolism, and transcription factors (Fig. 2.1.1–2.1.7). However, low temperature has also been shown to modulate kiwifruit ripening independently of ethylene (Mworia *et al*., 2012; Asiche *et al*., 2018). This conclusion was replicated in this study as 'Rainbow Red' kiwifruit ripening proceeded at

relatively lower temperatures than 22 ºC in the absence of detectable ethylene production and it was not suppressed by repeated 1-MCP treatments (Fig. 2.1.2). While multiple kiwifruit ripening-related genes have been shown to respond to storage at 5 ºC for 4 weeks (Asiche *et al*., 2018), it remains unclear how these genes are affected by different storage temperatures. This study demonstrated the ripening behaviour and expression pattern of associated genes at different storage temperatures and during on-vine ripening.

2.1.4.1. Dose × time-dependent fruit ripening during storage

During storage, healthy kiwifruit did not produce any detectable ethylene which could be attributed to the lack of expression changes in *AcACS1* and *AcACO2* (Fig. 2.1.4A, B). Both *AcACS1* and *AcACO2* have been linked with autocatalytic ethylene production during fruit ripening in kiwifruit (Yin *et al*., 2008; Atkinson *et al*., 2011; McAtee *et al*., 2015), which also agrees with the present study as they were exclusively induced by propylene treatment in correspondence with the observed ethylene burst (Fig. 2.1.1A). The inconsistency between elevated *AcACO3* transcript levels during low temperature storage (Fig. 2.1.4C) and the lack of detectable ethylene production could be explained by the lack of *AcACS1* expression. Nevertheless, *AcACO3* expression depicted a dose × time-dependent response to temperature, being upregulated highly at 5 ºC and 10 ºC within 4 weeks, moderately at 15 ºC after 8 weeks and no significant response at 22 ºC.

The dose × time-dependent response to temperature is also observed in the fruit softening and associated gene expression patterns. During storage at 5 ºC and 10 ºC, fruit softened to eating quality within 4 weeks (Fig. 2.1.2A), which could be attributed to increased expression levels of *AcXET2*, *AcEXP1*, *AcPG* and *AcPMEi* (Fig. 2.1.5B, D–F). However, fruit stored at 15 ºC required 8 weeks to attain eating quality firmness, and to elevate the expression levels of softening-related genes mentioned above. Particularly, *AcPG* expression was not affected by storage at 15 ºC (Fig. 2.1.5E), suggesting that 15 ºC did not provide an adequate low temperature stimulus for its modulation. Additionally, *AcXET2* expression was only upregulated during storage at 5 ºC (Fig. 2.1.5B), which suggested that it requires exceptionally low temperatures for its induction.

In this study, the expression of carbohydrate metabolism genes *AcSUS* and *Acβ-AMY1* increased during storage at all temperatures (Fig. 2.1.6A, B), which would account for the

observed SSC increase (Fig. 2.1.2B). Previous studies have also linked various *SUS* and *β-AMY* genes to sugar accumulation in multiple fruit (Moscatello *et al*., 2011; Richardson *et al*., 2011; Nardozza *et al*., 2013). It is however interesting that in kiwifruit, both *AcSUS* and *Acβ-AMY1* showed a dose × time-dependent response to temperature as they were highly upregulated at lower storage temperatures than at higher temperatures. This dose × time-dependent response to temperature was also depicted by *Acβ-AMY2*, whose expression levels were remarkably higher at 5 ºC than at 10 ºC and 15 ºC, with virtually no change at 22 ºC (Fig. 2.1.6C). The relatively low expression levels of carbohydrate metabolism genes at 15 ºC and 22 ºC would be compensated by higher protein accumulation and metabolic rates, which would account for the similar SSC levels at all storage temperatures.

Multiple studies have demonstrated that gibberellins are inhibitors of fruit ripening, especially in citrus fruit (Rodrigo and Zacarias, 2007; Rios *et al*., 2010). In this regard, Atkinson *et al*. (2011) reported that kiwifruit ripening is accompanied by increased expression of a *AcGA2ox* gene that is associated with the degradation of gibberellins. Asiche *et al*. (2018) also reported that two gibberellins degradation genes, *AcGA2ox1* and *AcGA2ox2* were upregulated during ethylene-dependent and low temperature-modulated fruit ripening in kiwifruit. These results are consistent with the finding in this study that *AcGA2ox2* expression levels dramatically increased during low temperature storage in kiwifruit (Fig. 2.1.6D). Interestingly, low temperature regulation of *AcGA2ox2* was in a dose × time-dependent response manner, as the transcript levels were higher at 5 ºC than at 10 ºC and 15 ºC, and very minimal at 22 ºC.

Fruit ripening is regulated at the molecular level by numerous TFs, which are proteins that bind to promoter sites of specific ripening-related genes resulting in their upregulation or repression (Giovannoni, 2007; Yin *et al*., 2012). In this study, *AcERF6* expression was exclusively induced by propylene treatment (Fig. 2.1.7A), indicating that this TF was specifically involved in regulating ethylene-dependent fruit ripening changes. This is consistent with previous findings in 'Sanuki Gold' kiwifruit (Asiche *et al*., 2018). *AcNAC3* and *AcNAC4* were regulated by either ethylene or low temperature whereas *AcMADS2* was exclusively regulated by low temperature (Fig. 2.1.7B–D). However, their response to low temperature was independent of ethylene and dose × time-dependent in that the expression levels were higher at 5 ºC than at 10 ºC and 15 ºC while no significant changes were recorded at 22 ºC.

2.1.4.2. On-vine fruit ripening is a response to low temperature

On-vine kiwifruit ripening has been reported to occur in the absence of any detectable ethylene (McAtee *et al*., 2015; Murakami *et al*., 2015; Richardson *et al*., 2011). However, it remains unclear whether this ripening behaviour is regulated by trace levels of system I ethylene or another unknown mechanism. In the present study, on-vine fruit ripening in 'Rainbow Red' kiwifruit was in close agreement with a gradual decrease in environmental temperatures (Fig. 2.1.3), suggesting that low temperature may be involved in these changes. This hypothesis is supported by two main findings in this study.

The first evidence is the finding that genes which were exclusively regulated by ethylene such as *AcACS1* and *AcACO2* (Fig. 2.1.4A, B), *AcXET1* and *AcPL2* (Fig. 2.1.5A, C), and *AcERF6* (Fig. 2.1.7C), showed no change in expression during on-vine fruit ripening. The rationale in this finding is that if the trace levels of system I ethylene were physiologically active, then these genes should be also upregulated during on-vine fruit ripening. However, their expression levels were unchanged throughout the on-vine fruit ripening duration, strongly suggesting that ethylene signalling was non-functional in this process.

The possible involvement of low temperature in on-vine fruit ripening is also supported by the finding that ripening-associated genes that were differentially regulated by low temperature during storage also exhibited an expression increase during on-vine fruit ripening. Therefore, it is plausible to suggest that kiwifruit attached to the vines have the ability to detect the seasonal drops in temperature that are characteristic of autumn. This response to low environmental temperatures triggers the expression of various genes associated with fruit softening, SSC increase and TA reduction, which are likely to be modulated by TFs such as *AcNAC3*, *AcNAC4* and *AcMADS2* (Fig. 2.1.7B–D).

2.1.5. Conclusion

Results presented in this study demonstrate the dose × time-dependent regulation of kiwifruit ripening by low temperature independently of ethylene. Fruit ripening, particularly softening, and expression of related genes occurred within just 4 weeks of storage at 5 ºC and 10 ºC. This implies that 5 ºC and 10 ºC provide a strong low temperature stimulus that appreciably shortens the ripening duration. On the other hand, the low temperature stimulus provided at 15 ºC is

relatively weaker compared to 5 ºC and 10 ºC, which results in a longer ripening duration of 8 weeks. Storage at 22 ºC cannot adequately trigger fruit ripening. Additionally, this study demonstrates that there are strong similarities between on-vine fruit ripening and low temperature-modulated fruit ripening, as well as the dissimilarities of the two with ethylene-dependent ripening. Therefore, it is most likely that on-vine kiwifruit ripening is also modulated by a low temperature signal independently of ethylene.

2.2. Fruit ripening responses to low temperature in two kiwifruit cultivars differing in maturity date and postharvest storability

Abstract

Kiwifruit exhibit a peculiar ripening pattern, as extensive softening occurs in the absence of any detectable ethylene. We previously demonstrated that this softening behaviour is regulated by low temperature independently of ethylene. However, there are no reports that provide comparisons of the ripening patterns among different kiwifruit cultivars at various storage temperatures. The purpose of this study was to compare the ripening responses and associated gene expression in 'Sanuki Gold' (*Actinidia chinensis* var. chinensis) and 'Hayward' (*Actinidia chinensis* var. deliciosa) fruit, two kiwifruit cultivars that differ in harvest maturity dates and postharvest storability, during storage at 5 °C, 10 °C, 15 °C and 22 °C. Fruit softening, SSC increase and reduction of TA occurred in the absence of any detectable ethylene, and treatment with an ethylene inhibitor 1-MCP failed to suppress the changes, suggesting that they were independent of ethylene. 'Sanuki Gold' fruit showed a higher sensitivity to low temperature supported by accelerated fruit softening and TA reduction, and induction of several genes such as *AcACO3*, *AcXET2*, *AcPG*, *AcEXP1*, *AcPMEi*, *AcGA2ox1*, *AcMADS2*, *AcNAC5* and *AcbZIP2* at 5 °C, 10 °C and 15 °C within 28 d. By contrast, 'Hayward' fruit exhibited a lower sensitivity to low temperature as accelerated softening, TA reduction and induction of most ripening-associated genes were recorded only at 5 °C and 10 °C. These differences in sensitivity to low temperature, between 'Sanuki Gold' and 'Hayward' fruit, would account for the dissimilarities observed in harvest maturity dates and postharvest storability.

2.2.1. Introduction

Fruit ripening encompasses highly coordinated physiological, biochemical and structural changes such as cell wall modifications, carbohydrate metabolism, pigment degradation and synthesis, and production of aroma volatiles (Klee and Giovannoni, 2011; Osorio *et al*., 2013). These changes are often orchestrated by the expression of several ripening-associated genes through a network of signalling pathways (Bouzayen *et al*., 2010). On the one hand, fruit ripening is considered beneficial since it results in soft edible fruit with desirable quality attributes. However, premature or excessive fruit ripening during storage is undesirable since

it leads to huge postharvest losses (Golden *et al*., 2014). An understanding of the controllers of fruit ripening aspects is essential for successful maintenance of postharvest quality.

Climacteric fruit ripening is largely driven by ethylene-regulated changes in gene expression (Giovannoni, 2004; Klee and Giovannoni, 2011; Xu *et al*., 2012). In kiwifruit (*Actinidia* spp.), exogenous ethylene (or its analogue, propylene) initiates rapid ripening-associated changes in a typical climacteric ripening pattern (Antunes *et al*., 2000; Mworia *et al*., 2010). Postharvest diseases (blossom-end rot, stem-end rot, and body rot) caused by *Botryosphaeria* sp., *Botrytis cinerea* and *Phomopsis* sp. are widely known for their devastating ethylene-related spoilage in kiwifruit during storage (Koh *et al*., 2005; Manning *et al*., 2016). Therefore, successful postharvest handling aims at ensuring no substantial ethylene accumulation in storage chambers (Atkinson *et al*., 2011). In addition, cold storage (0–4 °C) is universally used to extend the postharvest life of kiwifruit (Arpaia *et al*., 1987), based on the rationale that low temperature slows most cell metabolic activities, eventually delaying fruit ripening and senescence (McGlasson *et al*., 1979; Hardenburg *et al*., 1986).

During low temperature storage, kiwifruit exhibit a peculiar ripening behaviour in that extensive softening (to < 10 N) occurs in the absence of any detectable ethylene (Hewett *et al*., 1999; Kim *et al*., 1999). The mechanisms underlying the induction of fruit ripening in kiwifruit by low temperature remain elusive. Kiwifruit are considered highly sensitive to small ethylene concentrations (0.005–0.01 μLL^{-1}), which have been shown to cause softening and postharvest losses during cold storage (Kim *et al*., 1999; Antunes, 2007; Pranamornkith *et al*., 2012; Jabbar and East, 2016). Most of the attempts to explain the atypical softening of kiwifruit during cold storage attribute it to ethylene signalling based on the extrapolation of the above finding; that is, kiwifruit are highly sensitive to basal levels of ethylene (system I ethylene) present in most fruit (Kim *et al*., 1999; Yin *et al*., 2009). However, this hypothesis is based only on studies that were conducted at temperatures near 0 °C. Experiments comparing the ripening behaviour at different storage temperatures are required to uncover the mechanisms for extensive softening in kiwifruit during cold storage.

There are two popular kiwifruit cultivars in Japan; ‘Hayward’ (*Actinidia chinensis* var. deliciosa) and ‘Sanuki Gold’ (*Actinidia chinensis* var. chinensis). ‘Hayward’ fruit are late-maturing and are characterized by relatively low soluble solids concentration (SSC, 11–14 %)

and high titratable acidity (TA, ~1.5 %) when fully ripe (Asiche *et al*., 2016). 'Hayward' is the leading cultivar on the international market, and this is attributed to the high storability of the fruit; up to six months at 0 °C (Arpaia *et al*., 1987; Ritenour *et al*., 1999). On the other hand, 'Sanuki Gold' fruit are early-maturing and are considered premium because of their large size (200 g), high SSC (> 16 %) and low TA (1 %) when fully ripe (Fukuda *et al*., 2007). However, 'Sanuki Gold' fruit have very short storage life (one to two months) which poses a major problem during postharvest handling (Mworia *et al*., 2012; Asiche *et al*., 2016). Until now, the mechanisms underlying the discrepancy in storage life, between 'Sanuki Gold' and 'Hayward' fruit, remain unknown.

Previously, Mworia *et al*. (2012) demonstrated that softening in 'Sanuki Gold' kiwifruit occurred faster during storage at 4 °C than at 25 °C in the absence of any detectable ethylene, which was linked to increased accumulation of *polygalacturonase* (*AcPG*), *pectate lyase* (*AcPL*) and *expansin* (*AcEXP*) mRNAs. These changes were not suppressed by repeated exposure of kiwifruit to 1-methylcyclopropene (1-MCP), a potent ethylene perception inhibitor, suggesting that the induction of kiwifruit ripening by low temperature was independent of ethylene signalling. This conclusion was further corroborated by our recent findings, through comparative transcriptome analyses in 'Sanuki Gold' fruit, that kiwifruit possess a distinct set of genes that are exclusively regulated by low temperature (5 °C) independent of ethylene (Asiche *et al*., 2018). While ethylene-independent modulation of ripening by low temperature has also been previously reported in other kiwifruit cultivars such as 'Rainbow Red' and 'Hayward' (Asiche *et al*., 2017; Mitalo *et al*., 2018), there are no reports that compare the ripening responses of different cultivars at various storage temperatures.

In this regard, the present study compared the ripening behaviour of 'Sanuki Gold' and 'Hayward' kiwifruit cultivars during storage at four different temperature conditions (5 °C, 10 °C, 15 °C and 20 °C) in the absence of detectable ethylene. The expression pattern of selected ripening-associated genes in these two cultivars during storage was also studied. The overall objective was to determine whether there are dissimilarities that could account for the discrepancies observed in harvest maturity dates and postharvest storability between 'Sanuki Gold' and 'Hayward' fruit.

2.2.2. Materials and methods

2.2.2.1. Plant material

'Sanuki Gold' kiwifruit (500 fruit) were harvested on 9th October 2014 corresponding to 151 days after full bloom (DAFB) from a commercial orchard in Takamatsu, Japan. 'Hayward' kiwifruit (500 fruit) were harvested at 169 DAFB (27th October 2014) from an experimental orchard at Okayama University, Okayama, Japan. Upon harvesting, fruit were immediately transported to the Laboratory of Postharvest Physiology at Okayama University and sorted to ensure uniform size, absence of defects, and no detectable ethylene production.

2.2.2.2. Treatments

All fruit were dipped in a mixed fungicide solution containing 0.015 g L^{-1} of oxytetracycline (Pfizer Co., Ltd., Japan), 0.15 g L^{-1} of streptomycin (Pfizer), 0.5 g L^{-1} of iprodione (FMC Chemicals Ltd., Japan), *Bacillus subtillis* HAI0404 spores (1 x 10^{10} cfu/L, Nippon Soda Co., Ltd., Japan), and 0.5 g L^{-1} of benomyl (Sumitomo Chemical Ltd., Japan). After air-drying, fruit were divided into four groups for storage at either 5 °C (40 fruit), 10 °C (40 fruit), 15 °C (40 fruit) or 22 °C (130 fruit) in air (ethylene-free chambers). Separately, four other groups (containing the same number of fruit as the corresponding temperature) were treated with 1-MCP (5 µL L^{-1}, 12 h) twice a week to keep the fruit insensitive to ethylene (Mworia *et al*., 2012). 1-MCP was released by dissolving 1-MCP powder (SmartFresh^{SM}, AgroFresh, PA, USA) in water.

2.2.2.3. Storage technique

During storage, fruit were individually wrapped in perforated polythene bags to reduce water loss, and then placed separately (10 cm apart) in plastic crates (Asiche *et al*., 2018). This storage technique allowed for the monitoring of ethylene production pattern of each fruit, unlike the conventional technique where ethylene emanating from a single fruit can influence adjoining fruit. To avoid accumulation of ethylene in the storage chambers, fruit that produced detectable ethylene (≥ 0.003 ng kg^{-1} s^{-1}) were removed and monitored separately.

2.2.2.4. Determination of ethylene production

Ethylene production was measured by incubating individual fruit in 440-mL airtight containers. After about 1 h, 1 mL of headspace gas was withdrawn and injected into a gas chromatograph

(Model GC8 CMPF; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (set at 200 °C) and an activated alumina column set at 80 °C (Mworia *et al*., 2010). This procedure has a minimum ethylene detection capacity of 0.003 ng kg^{-1} s^{-1}.

2.2.2.5. Fruit quality assessments

Firmness, SSC and TA were monitored using fruit that did not produce any detectable ethylene (five independent biological replicates with three technical replicates) at harvest (0 d) and after 28 d and 56 d of storage. Core and outer pericarp firmness were measured using a penetrometer (model SMTT50; Toyo Baldwin, Tokyo, Japan) fitted with a 5-mm plunger (Mworia *et al*., 2012). SSC of the fruit juice was measured using a digital Atago PR1 refractometer (Atago Co. Ltd, Tokyo, Japan), and the value was expressed as a percentage. TA was determined by titrating the fruit juice against 0.1N NaOH using phenolphthalein as a pH indicator, and the value was expressed as percentage citric acid equivalents. The outer pericarp of fruit (three independent biological replicates for each treatment) was then cut into small pieces and stored at -80 °C until further analysis.

2.2.2.6. Effect of propylene on kiwifruit ripening

The effect of propylene exposure on kiwifruit ripening was reported in our previous study (Asiche *et al*., 2018). Kiwifruit at commercial maturity were continuously exposed to 5000 µL L^{-1} propylene at 20 °C to induce ripening-associated changes, and endogenous ethylene production (McMurchie *et al*., 1972; Mworia *et al*., 2010).

2.2.2.7. RNA extraction, cDNA synthesis and RT-qPCR analysis

Total RNA was extracted from the outer pericarp (three biological replicates) of fruit at 0 d (harvest), 5 d (for propylene treatment), and 28 d (for storage tests) using a method for polysaccharide-rich fruit with slight modifications (Ikoma *et al*., 1996). The extracted RNA was treated with DNase I followed by clean-up using FavorPrep After TriReagent RNA Clean-up kit (Favorgen Biotech Co., PingTung, Taiwan). First strand cDNA was synthesized from 2.4 µg of RNA using RevTra Ace reverse transcriptase (Toyobo, Osaka, Japan) and random hexamer primer according to the manufacturer's instructions.

Eighteen genes were selected from a list of genes that showed differential expression in response to ethylene and low temperature, previously reported in 'Sanuki Gold' fruit (Asiche

et al., 2018). Out of these, seven genes were exclusively regulated by ethylene, seven were exclusively regulated by low temperature and the remaining four were regulated by either ethylene or low temperature. *1-aminocyclopropane-1-carboxylic acid* (*ACC*) *synthase 1* (*AcACS1*) and *ACC oxidase 2* (*AcACO2*) have been previously linked to autocatalytic ethylene production in kiwifruit (McAtee *et al*., 2015; Atkinson *et al*., 2011), while *AcACO3* was identified in our previous study (Asiche *et al*., 2018). *Ethylene response 2* (*AcETR2*) is 100 % homologous to *ETR2* previously reported in 'Hort16A' kiwifruit (McAee *et al*., 2015). Softening-related genes included *AcPG* that is highly homologous to *PG-C* (Wang *et al*., 2000; AAF71158) extracted from *Actinidia chinensis* tissues, *AcEXP1* (*AC-EXP*: AY390358; Mworia *et al*., 2012), *pectin methyl esterase inhibitor AcPMEi* (*PMEi*: FG458520; Atkinson *et al*., 2011), *AcPL1* (*PL1*: HQ108112) isolated from *A. deliciosa*, and *xyloglucan endotransglycosylase AcXET1* (*XET5*: EU494950) and *AcXET2* (*XET10*: EU494955; Atkinson *et al*., 2009). *B-Amylase 3L* (*AcBAM3L*) is 100 % homologous to *BAM3* (KX383649; Hu *et al*., 2016), *AcBAM3* is closely related to *BAM3* (FG455287; Nardozza *et al*., 2013) while *AcGA2ox1* is 100 % homologous to *Gibberellin-2-oxidase* (FG471283; Atkinson *et al*., 2011). The *ethylene response factor* (*AcERF6*) was previously reported in 'Hayward' kiwifruit (GQ869857; Yin *et al*., 2010). The other transcription factor genes including *AcNAC1*, *AcNAC5* (*no apical meristem [NAM], Arabidopsis transcription activation factor [ATAF], and cup-shaped cotyledon [CUC])-domain*), *AcMADS2* (*MADS-box domain*) and *AcbZIP2* (*basic leucine zipper 2*) are novel genes that were recently identified in 'Sanuki Gold' fruit (Asiche *et al*., 2018).

Gene-specific primers for RT-qPCR analysis were designed using Primer3 software (version 0.4.0, http://bioinfo.ut.ee/primer3-0.4.0/) (Table 2.2.1). The RT-qPCR mixture comprised 10 µL of SYBR Green Premix EX Taq II (TaKaRa, Otsu, Japan), 1.6 µL of each primer, 4 µL of cDNA, 4.2 µL of RNase-free water and 0.2 µL of FITC. The reaction was performed using MyiQ Single Color Real-Time PCR detection system (BioRad, Hercules, CA), according to the manufacturer's instructions. The cycling condition was set at 95 °C for 5 min, denaturation at 95 °C for 5 s, and annealing/extension at 59 °C for 10 s (45 cycles). The specificity of the PCR products was verified by dissociation (melting) curve analysis.

Gene expression was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$) with *AcActin* as the housekeeping gene, and fruit at 0 d set as 1. In our most recent report, RNA-seq revealed five genes that were annotated as Actin, all of which were constitutively expressed at both 5 °C and

20 °C as well as in propylene-treated fruit (Asiche *et al*., 2018). Thus, *AcActin* was an appropriate reference gene for RT-qPCR analysis of the selected genes.

Table 2.2.1. Kiwifruit primers used for RT-qPCR analysis. Gene sequences were obtained from the Kiwifruit Genome and NCBI Databases, and primers were designed using Primer3 software (version 0.4.0, http://bioinfo.ut.ee/primer3-0.4.0/).

S/N	Gene	Name	Access. code	Orientation	Sequence
1	*AcACS1*	ACC Synthase 1	Achn364251	Fw (5' to 3')	GAAAGGCTGCGTGCAATTCTC
				Rv (3' to 5')	CCTGAAAATGGACTGCCCATC
2	*AcACO2*	ACC Oxidase 2	Achn326461	Fw (5' to 3')	TCTCAGAAATCCCCGATCTCG
				Rv (3' to 5')	TTGGAGCCACTGAAAGCCTTC
3	*AcACO3*	ACC Oxidase 3	Achn150611	Fw (5' to 3')	CAGATGGCAACAGAATGTCG
				Rv (3' to 5')	AACTTGAGGCCAGCATAGAG
4	*AcETR2*	Ethylene response 2	Achn067861	Fw (5' to 3')	ATGCCAATGCTTCAGGTTTC
				Rv (3' to 5')	GATCCCTCATATGCTGGGAC
5	*AcPG*	Polygalacturonase	Achn051381 (AF152756)	Fw (5' to 3')	TGGATTTGTTAGGGGTGTGC
				Rv (3' to 5')	CAACTTGTGTCGCTGATGAC
6	*AcPL1*	Pectate lyase 1	Achn070291	Fw (5' to 3')	TCAACAATGGCGGATAGGTCA
				Rv (3' to 5')	CGAATGCTCACGTCAACCATG
7	*AcXET1*	Xyloglucan Endotransglycosylase 1	Achn349851	Fw (5' to 3')	CGTTCGATCAACCGATGAAGA
				Rv (3' to 5')	TCTTGTAAGCAGCCACGAAGG
8	*AcXET2*	Xyloglucan endotransglycosylase 2	Achn023381	Fw (5' to 3')	GCTTCTCACGCTTTCTCTCG
				Rv (3' to 5')	GTTGACCCCAACGAAGACAG
9	*AcEXP1*	Expansin 1	Achn336951 (AY390358)	Fw (5' to 3')	CGTGCTTCGAGCTAAAGTGC
				Rv (3' to 5')	CGGCGATCTTGAGGAACATG
10	*AcPMEi*	Pectin methyl esterase inhibitor	Achn319051 (FG458520)	Fw (5' to 3')	CTTTGCATCACCTCGCTCTC
				Rv (3' to 5')	ACTGGGACAGCTTCACCATC
11	*AcBAM3L*	β–Amylase 3L	Achn141771 (KX383649)	Fw (5' to 3')	CCCCACATTGATGGAATGAC
				Rv (3' to 5')	GTTTGTGATGCTGCCACTCG
12	*AcBAM3*	β–Amylase 3	Achn212571 (FG455287)	Fw (5' to 3')	CAGAGAACGCAAACTGCTCG
				Rv (3' to 5')	GTTCCCGGAGTCTGATCTAC
13	*AcGA2ox1*	Gibberellic acid oxidase 1	Achn209941	Fw (5' to 3')	AAGGTGGCCCTCCAAAATAG
				Rv (3' to 5')	GTCCCTCCTGACCAGGATTC
14	*AcERF6*	Ethylene response factor 6	GQ869857	Fw (5' to 3')	ACGGCATCGAAAACCGTTC
				Rv (3' to 5')	TGCCGGATTCCTCTGTACTTG
15	*AcMADS2*	MADS-box transcription factor 2	Achn235371	Fw (5' to 3')	GGACAAGAACAGTCGCCAGG
				Rv (3' to 5')	GTATCTGTCGCCGGTGATG
16	*AcNAC1*	NAM, ATAF and CUC transcription factor 1	Achn104221	Fw (5' to 3')	ATACCACGGACAGCAAATTC
				Rv (3' to 5')	TGGTGGTGAAACAGAAAGCC
17	*AcNAC5*	NAM, ATAF and CUC transcription factor 5	Achn169681	Fw (5' to 3')	GGTTATTGGAAGGCCACTGG
				Fw (3' to 5')	CCATCAACAAGGCGATATTC
18	*AcbZIP2*	Basic leucine zipper transcription factor 2	Achn227711	Fw (5' to 3')	GGTCAGCGCACAGGATTGAG
				Rv (3' to 5')	AGCCAGATTTGATTGCTTGG
19	*AcActin*	Actin	EF063572	Fw (5' to 3')	TGGAATGGAAGCTGCAGGA
				Rv (3' to 5')	CACCACTGAGCACAATGTTGC

2.2.2.8. Statistical analysis

Physiological data generated from this study were subjected to multivariate analysis of variance (MANOVA) using BellCurve for Excel (version 2.13) provided by Social Survey Research Information Co., Ltd. Gene expression data were subjected to ANOVA analysis followed by post-hoc Tukey tests ($p < 0.05$) using the Agricolae package in R software (version 3.3.2). Standard errors were calculated using Microsoft Excel.

2.2.3. Results

2.2.3.1. Fruit quality at harvest

At harvest, 'Sanuki Gold' kiwifruit had a high core and flesh firmness (126 N and 55 N respectively), low SSC (8.6 %) and high TA (2.58 %) (Fig. 2.2.3A–D). 'Hayward' kiwifruit also had high core and flesh firmness (176 N and 73 N respectively), low SSC (7 %) and high TA (2.6 %) (Fig. 2.2.3E–H). Overall, fruit of both cultivars were at a commercial maturity stage at the time of harvest, with SSC > 6 % and producing no detectable ethylene, consistent with previous reports (Snelgar *et al*., 1993; Salinero *et al*., 2009; Richardson *et al*., 2011).

2.2.3.2. Ethylene production and fruit rot incidence

Ethylene measurements were repeatedly conducted for each fruit during storage. Fruit that produced detectable ethylene (≥ 0.003 ng kg^{-1} s^{-1}) were removed from storage chambers and monitored separately. These fruit showed a burst of ethylene production, and developed rot symptoms within a few days after removal from storage chambers (Fig. 2.2.1). The proportion of fruit that produced detectable ethylene was highest during storage at 22 °C and after 56 d, it was about 80 % and 90 % for 'Sanuki Gold' and 'Hayward' cultivars respectively (Fig. 2.2.2). However, this was significantly reduced to < 15 % and < 37.5 % for 'Sanuki Gold' and 'Hayward' fruit, respectively during storage at 10 °C and 15 °C, whereas it was virtually zero at 5 °C. Notably, a considerable proportion of fruit did not produce any detectable ethylene during the entire storage period (for all temperature conditions), and these were used for fruit quality assessments.

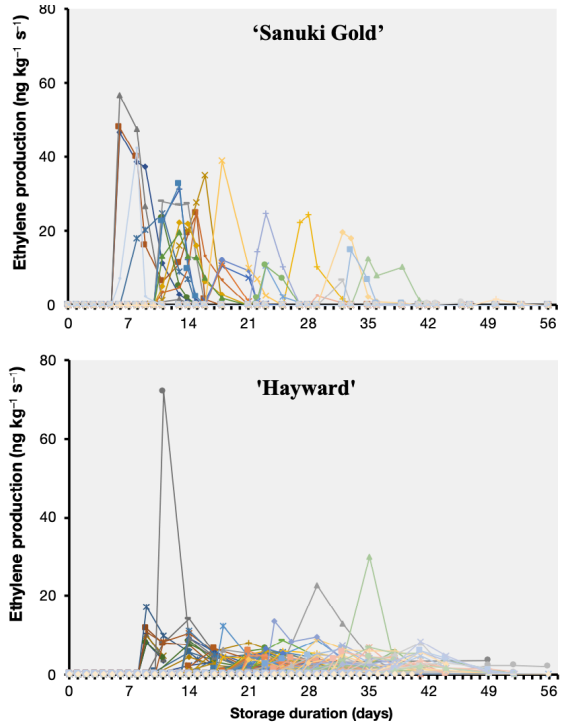


Fig. 2.2.1. Ethylene production pattern of **(A)** 'Sanuki Gold' and **(B)** 'Hayward' kiwifruit that were removed from storage chambers upon initiation of detectable ethylene production (≥ 0.003 ng kg^{-1} s^{-1}). These fruit softened rapidly and developed rot symptoms within a few days after removal from storage chambers.

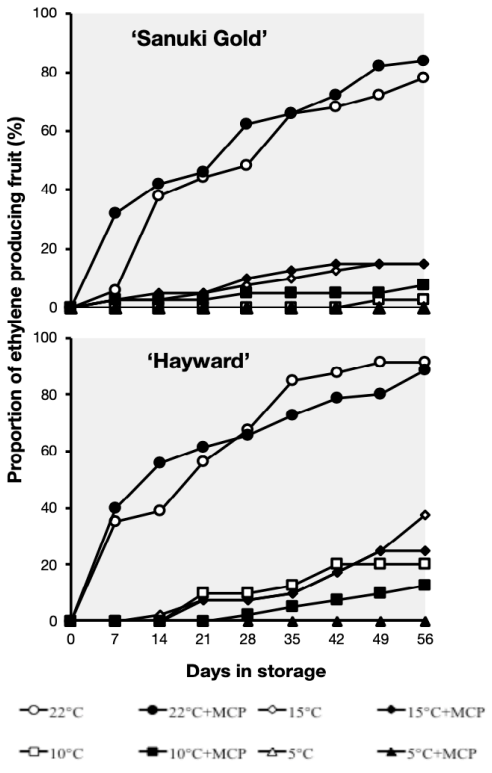


Fig. 2.2.2. Proportion of fruit that produced detectable ethylene (≥0.003 ng kg^{-1} s^{-1}). during storage of 'Sanuki Gold' and 'Hayward' kiwifruit at various temperatures.

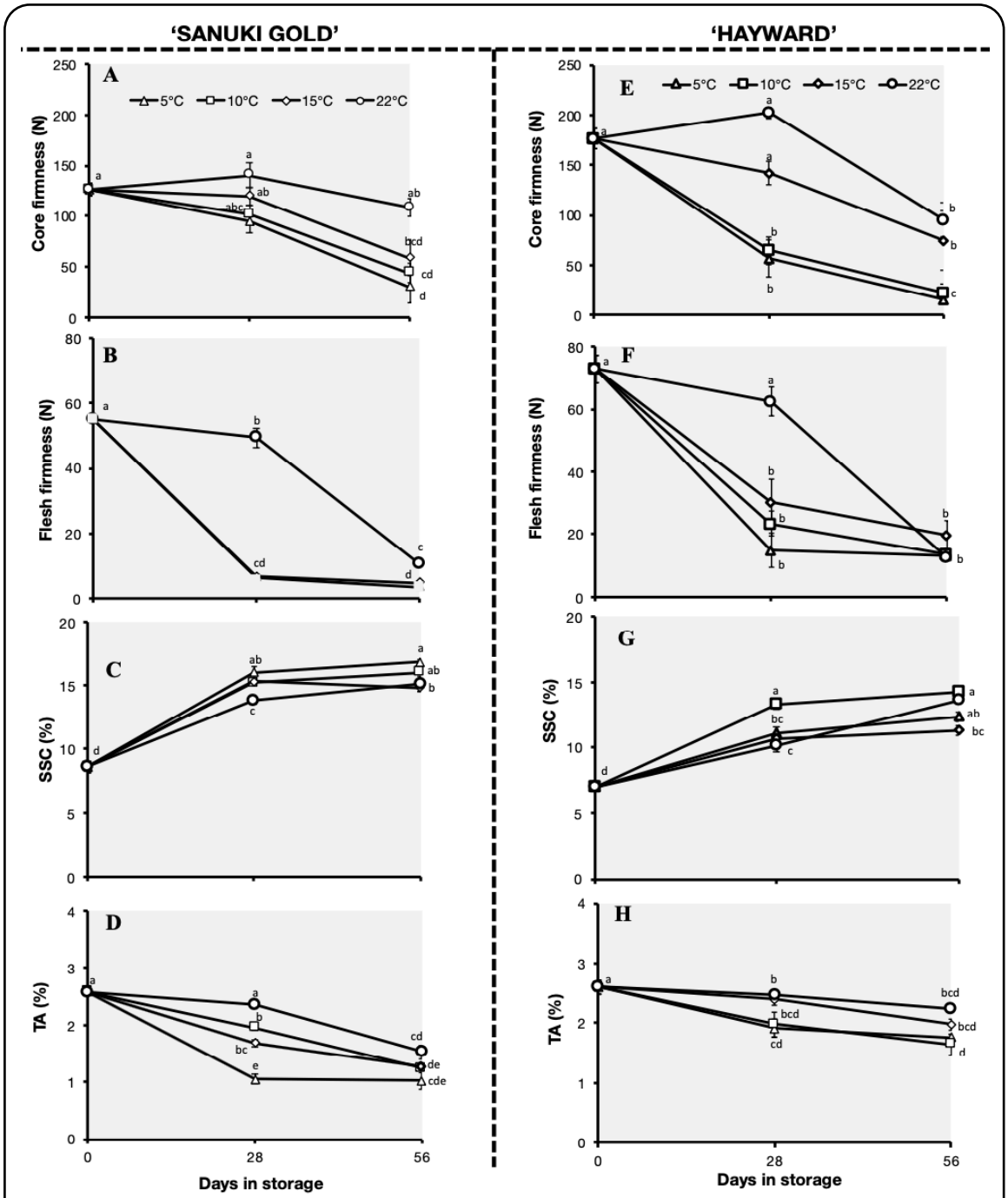


Fig. 2.2.3. Fruit ripening characteristics of 'Sanuki Gold' (A, B, C, D) and 'Hayward' (E, F, G, H) kiwifruit during storage at various temperatures. Measurements were done using fruit that did not produce any detectable ethylene. Each data set represents the mean ± SE of five independent biological replicates with three technical replicates. Different letters indicate statistically significant differences ($p \leq 0.05$).

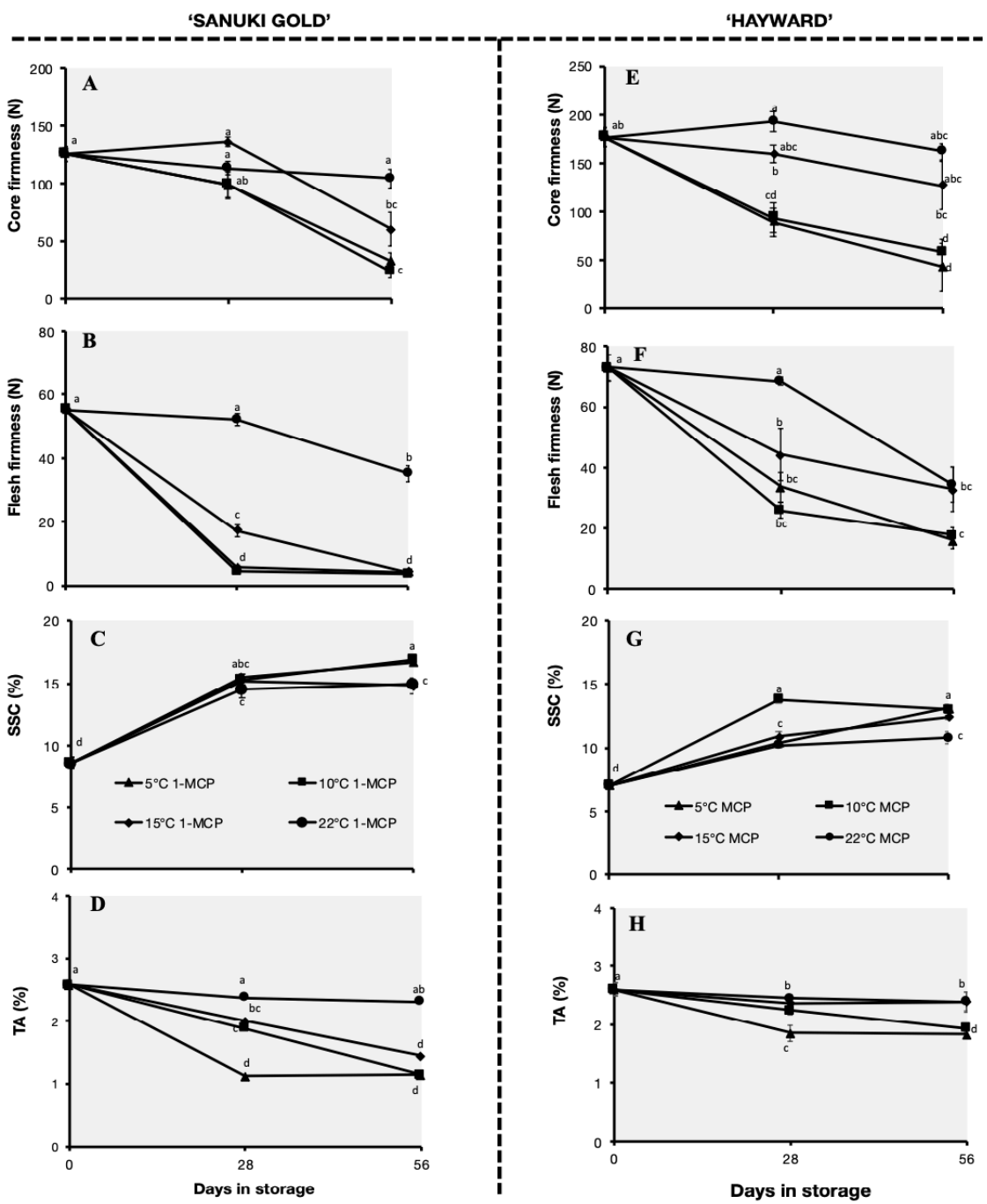


Fig. 2.2.4. Ripening characteristics of 1-MCP treated fruit of 'Sanuki Gold' (A, B, C, D) and 'Hayward' (E, F, G, H) cultivars during storage at various temperatures in the absence of detectable ethylene. Kiwifruit at commercial maturity were regularly treated with 1-MCP (5 µL L^{-1}, 12 h) twice a week during storage at either 5 °C, 10 °C, 15 °C or 22 °C. Core firmness, flesh firmness, soluble solids concentration (SSC) and titratable acidity (TA) were determined using kiwifruit that did not produce any detectable ethylene. Each data set represents the mean ± SE of five independent biological replicates with three technical replicates. Different letters indicate statistically significant differences ($p \leq 0.05$).

Table 2.2.2. Multivariate analysis of variance (MANOVA) results of the changes in core firmness, flesh firmness, SSC and TA during storage of 'Sanuki Gold' and 'Hayward' fruit at 5 ℃, 10 ℃, 15 ℃ and 22 ℃. Statistical analysis was done using BellCurve for Excel (version 2.13) provided by Social Survey Research Information Co., Ltd.

Effect		Value	F	df1	df2	P value	* : P<0.05 ** : P<0.01
Cultivar	Pillai's trace	0.8732	105.0562	4	61	P < 0.001	**
	Wilks' lambda	0.1268	105.0562	4	61	P < 0.001	**
	Hotelling's trace	6.8889	105.0562	4	61	P < 0.001	**
	Roy's largest root	6.8889	105.0562	4	61	P < 0.001	**
Temperature	Pillai's trace	1.4796	15.3277	12	189	P < 0.001	**
	Wilks' lambda	0.0867	20.4780	12	161.6823326	P < 0.001	**
	Hotelling's trace	4.7951	23.8422	12	179	P < 0.001	**
	Roy's largest root	3.4153	53.7915	4	63	P < 0.001	**
Duration	Pillai's trace	0.7048	36.4058	4	61	P < 0.001	**
	Wilks' lambda	0.2952	36.4058	4	61	P < 0.001	**
	Hotelling's trace	2.3873	36.4058	4	61	P < 0.001	**
	Roy's largest root	2.3873	36.4058	4	61	P < 0.001	**
Cultivar * Temperature	Pillai's trace	0.7371	5.1300	12	189	P < 0.001	**
	Wilks' lambda	0.3949	5.6697	12	161.6823326	P < 0.001	**
	Hotelling's trace	1.2139	6.0359	12	179	P < 0.001	**
	Roy's largest root	0.8941	14.0814	4	63	P < 0.001	**
Cultivar * Duration	Pillai's trace	0.1628	2.9662	4	61	0.0264	*
	Wilks' lambda	0.8372	2.9662	4	61	0.0264	*
	Hotelling's trace	0.1945	2.9662	4	61	0.0264	*
	Roy's largest root	0.1945	2.9662	4	61	0.0264	*
Temperature * Duration	Pillai's trace	1.0124	8.0219	12	189	P < 0.001	**
	Wilks' lambda	0.2058	11.0176	12	161.6823326	P < 0.001	**
	Hotelling's trace	2.8229	14.0361	12	179	P < 0.001	**
	Roy's largest root	2.4051	37.8803	4	63	P < 0.001	**
Cultivar * Temperature * Duration	Pillai's trace	0.6243	4.1392	12	189	P < 0.001	**
	Wilks' lambda	0.4620	4.5664	12	161.6823326	P < 0.001	**
	Hotelling's trace	0.9841	4.8930	12	179	P < 0.001	**
	Roy's largest root	0.7685	12.1042	4	63	P < 0.001	**

2.2.3.3. Ripening behaviour of 'Sanuki Gold' and 'Hayward' kiwifruit cultivars at different storage temperatures

Figure 2.2.3 shows the changes in different ripening characteristics of 'Sanuki Gold' and 'Hayward' kiwifruit producing no detectable ethylene during storage at various temperatures. The core firmness of 'Sanuki Gold' fruit showed no significant changes during storage at 22 °C, while it decreased from the initial 126 N at 0 d to 30 N at 5 °C, 44 N at 10 °C and 59 N at 15 °C after 56 d (Fig. 2.2.3A). In 'Hayward' fruit, a significant decrease in core firmness was observed at all storage temperatures although it was rather faster at 5 °C and 10 °C compared to 15 °C and 22 °C (Fig. 2.2.3E). The flesh (outer pericarp) firmness significantly decreased in fruit of both cultivars irrespective of storage temperature (Fig. 2.2.3B, F). However, in 'Sanuki Gold' fruit, the decrease in flesh firmness was faster during storage at 5 °C, 10 °C and 15 °C from the initial 55 N at 0 d to 6 N at 28 d, and 3 N at 56 d, compared to 49 N and 10 N at 28 d and 56 d respectively for fruit at 22 °C (Fig. 2.2.3B). Similarly, a significantly faster decrease in flesh firmness was recorded in 'Hayward' fruit at 5 °C, 10 °C and 15 °C compared to 22 °C (Fig. 2.2.3F). Although there were no statistically significant differences in the flesh firmness of 'Hayward' fruit stored at 5 °C, 10 °C and 15 °C for 28 d, flesh firmness was substantially higher at 15 °C (30 N) compared to 10 °C (23 N) and 5 °C (15 N) (Fig. 2.2.3F).

There was a significant SSC increase in fruit of both cultivars regardless of storage temperature (Fig. 2.2.3C, G). In 'Sanuki Gold' fruit, SSC increased from 8.6 % at 0 d to > 15 % at 56 d at all storage temperatures (Fig. 2.2.3C). SSC also increased in 'Hayward' fruit at all storage temperatures from 7 % at harvest to 11.3–14 % after 56 d, although the increase was particularly faster at 10 °C compared to other storage temperatures (Fig. 2.2.3G). A significant reduction of TA was recorded in 'Sanuki Gold' fruit regardless of storage temperature, although the reduction was faster at 5 °C from 2.58 % at 0 d to 1 % at 56 d compared to 10 °C and 15 °C (1.26 %); fruit at 22 °C registered significantly high TA (1.53 %) at the same sampling time (Fig. 1D). For 'Hayward' fruit, TA reduction occurred faster during storage at 5 °C and 10 °C from 2.6 % at 0 d to about 1.7 % after 56 d, whereas TA changes were insignificant at 15 °C and 22 °C (Fig. 2.2.3H).

Overall, fruit of 'Sanuki Gold' and 'Hayward' cultivars showed a differential ripening response across storage temperature. While softening and TA reduction was accelerated in 'Sanuki Gold'

fruit at 5 °C, 10 °C and 15 °C, 'Hayward' kiwifruit showed accelerated softening and TA reduction during storage at 5 °C and 10 °C. Similar results were observed in fruit regularly treated with 1-MCP, suggesting that the changes were independent of ethylene (Fig. 2.2.4). A multivariate analysis of variance (MANOVA) using either of the tests (Pillai's trace, Wilk's lambda, Hotelling's trace and Roy's largest root) showed a significant difference in ripening profiles between the two cultivars ($p < 0.01$) (Table 2.2.2).

2.2.3.4. Expression analysis of ripening-associated genes in 'Sanuki Gold' and 'Hayward' kiwifruit during storage

The expression of two genes associated with ethylene biosynthesis, *AcACS1* and *AcACO2*, consistently increased in propylene-treated fruit of both 'Sanuki Gold' and 'Hayward' cultivars with minimal or no changes during storage (Fig. 2.2.5A, B, E, F). This was consistent with the presence of detectable endogenous ethylene production in propylene-treated fruit (Appendix 1), and the lack of it during storage. By contrast, the expression of a third ethylene biosynthesis-related gene, *AcACO3*, showed no significant changes in propylene-treated fruit, indicating that it is not involved in autocatalytic ethylene biosynthesis (Fig. 2.2.5C, G). During storage, *AcACO3* expression remarkably increased in both 'Sanuki Gold' and 'Hayward' fruit at 5 °C, 10 °C and 15 °C while changes at 22 °C were insignificant. However, while 'Sanuki Gold' fruit at 5 °C, 10 °C and 15 °C showed almost similar expression levels of *AcACO3* (> 27-fold), the expression in 'Hayward' fruit was considerably high at 5 °C (412-fold) compared to 10 °C (53-fold) and 15 °C (19-fold). Lastly, the expression of *AcETR2* was consistently high in propylene-treated fruit of both cultivars (Fig. 2.2.5D, H). However, during storage, increased expression of *AcETR2* was recorded only in 'Sanuki Gold' fruit at 5 °C, 10 °C and 15 °C while changes in 'Hayward' fruit were insignificant.

We also compared the expression patterns of six genes encoding cell wall modifying enzymes during kiwifruit storage (Fig. 2.2.6). Two of these, *AcPL1* and *AcXET1*, registered considerably high expression levels in propylene–treated fruit of both 'Sanuki Gold' and 'Hayward' cultivars, while their expression during storage was insignificant (Fig. 2.2.6A, B, G, H). Propylene treatment also induced the expression of *AcPG* and *AcEXP1* in fruit of both cultivars (Fig. 4D, E, J, K), and *AcPMEi* in 'Hayward' fruit (Fig. 2.2.6L). During storage, 'Sanuki Gold' fruit at 5 °C, 10 °C and 15 °C registered significantly high expression levels of *AcXET2*, *AcPG*, *AcEXP1* and *AcPMEi* compared to 22 °C (Fig. 2.2.6C, D, E, F). 'Hayward' fruit stored at 5 °C,

10 °C and 15 °C also showed increased expression of *AcPMEi*, compared to 22 °C (Fig. 2.2.6L). However, the expression of *AcPG* and *AcEXP1* was significantly high in 'Hayward' fruit stored at 5 °C and 10 °C while no significant changes were observed at 15 °C and 22 °C (Fig. 2.2.6J, K). There were no significant changes in expression of *AcXET2* during storage of 'Hayward' fruit (Fig. 2.2.6I).

Sugar accumulation during fruit ripening is due to the conversion of starch to simple sugars. The expression of a starch degradation–related gene, *AcBAM3L*, was consistently high in propylene-treated fruit of both cultivars (Fig. 2.2.7A, D). There was also a slight increase in *AcBAM3L* expression in 'Sanuki Gold' fruit at 15 °C, whereas no significant expression changes were recorded in 'Hayward' fruit during storage. A second starch degradation-associated gene, *AcBAM3* did not show any significant expression changes in propylene-treated 'Sanuki Gold' fruit, while it was slightly induced in propylene-treated 'Hayward' fruit (Fig. 2.2.7B, E). During storage, *AcBAM3* expression remarkably increased in fruit of both cultivars at 5 °C, 10 °C and 15 °C. Propylene treatment induced the expression of *AcGA2ox1* (gibberellic acid oxidase) in fruit of both cultivars (Fig. 2.2.7C, F). However, during storage, *AcGA2ox1* expression only increased in 'Sanuki Gold' fruit at 5 °C, 10 °C and 15 °C.

Finally, the expression of five genes encoding ripening-associated transcription factors was compared between 'Sanuki Gold' and 'Hayward' kiwifruit during storage (Fig. 2.2.8). Both the expression of *AcERF6* and *AcNAC1* increased in propylene-treated fruit of both cultivars, while no significant changes were observed during storage (Fig. 2.2.8A, C, F, H). The expression of *AcNAC5* was also induced in propylene-treated fruit of both cultivars (Fig. 2.2.8D, I). During storage, diverse expression patterns were observed between the fruit of 'Sanuki Gold' and 'Hayward'. 'Sanuki Gold' fruit registered significantly high expression of *AcMADS2* at 5 °C and 10 °C (Fig. 2.2.8B), as well as *AcNAC5* and *AcbZIP2* at 5 °C, 10 °C and 15 °C (Fig. 2.2.8D, E). On the other hand, in 'Hayward' fruit, a significant increase in expression was only observed for *AcMADS2* and *AcNAC5* at 5 °C (Fig. 6G, I), and *AcbZIP2* at 5 °C, 10 °C and 15 °C (Fig. 2.2.8J).

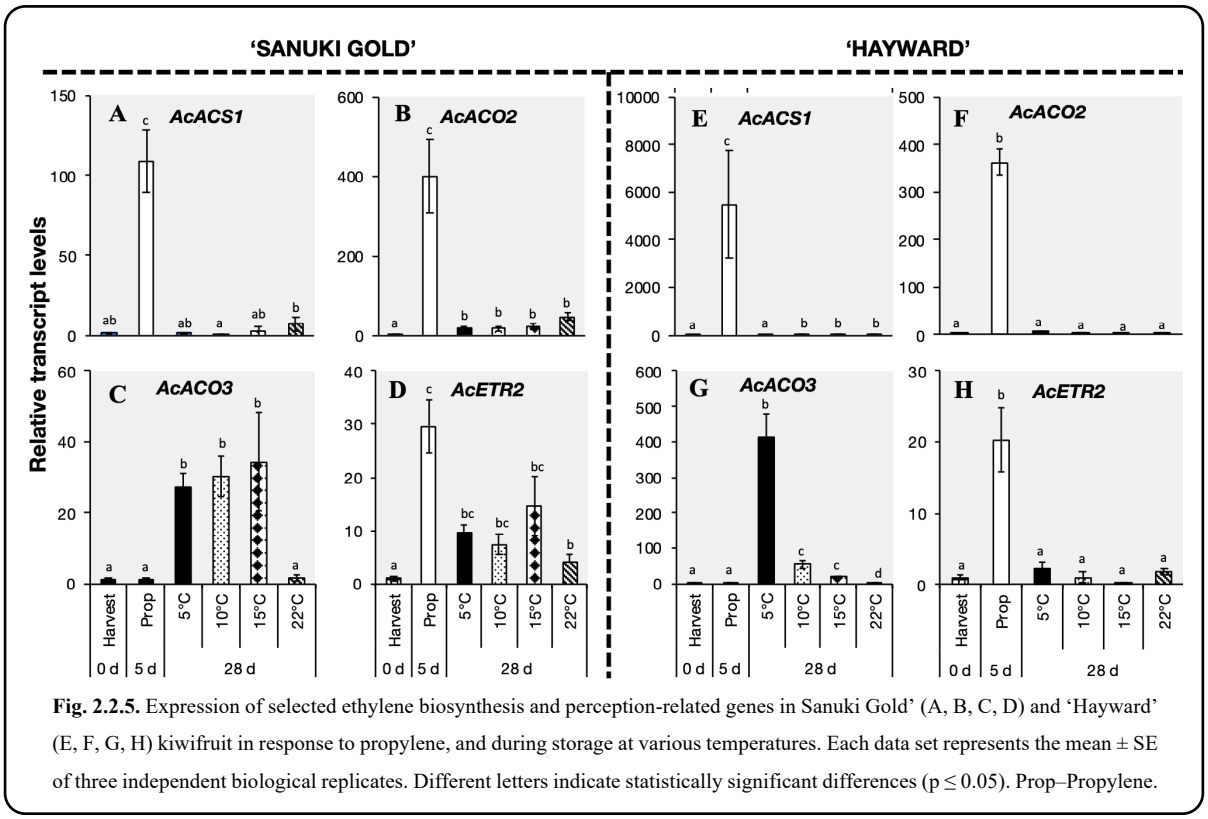


Fig. 2.2.5. Expression of selected ethylene biosynthesis and perception-related genes in Sanuki Gold' (A, B, C, D) and 'Hayward' (E, F, G, H) kiwifruit in response to propylene, and during storage at various temperatures. Each data set represents the mean ± SE of three independent biological replicates. Different letters indicate statistically significant differences ($p \leq 0.05$). Prop–Propylene.

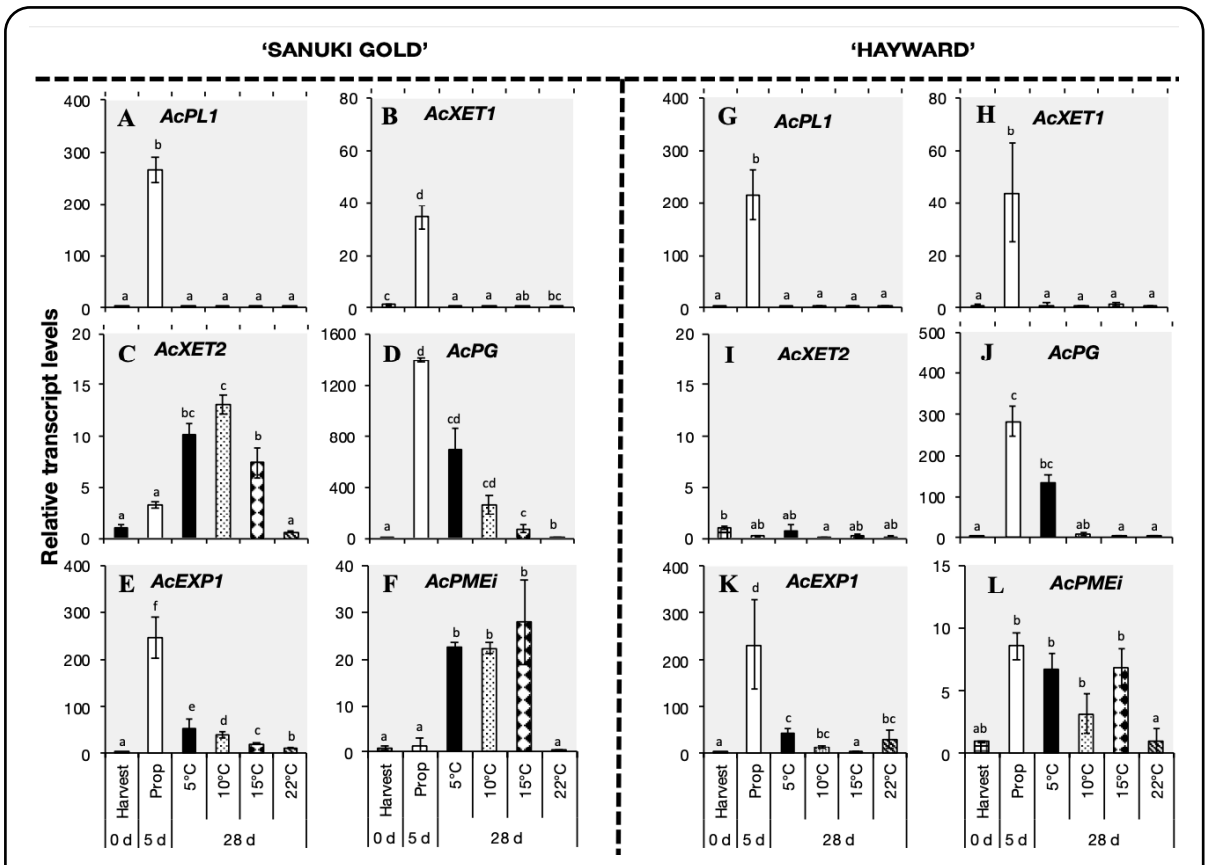


Fig. 2.2.6. Expression of selected cell wall modifying genes in 'Sanuki Gold' (A, B, C, D, E, F) and 'Hayward' (G, H, I, J, K, L) kiwifruit in response to propylene, and during storage at various temperatures. Each data set represents the mean ± SE of three independent biological replicates. Different letters indicate statistically significant differences ($p \leq 0.05$). Prop–Propylene.

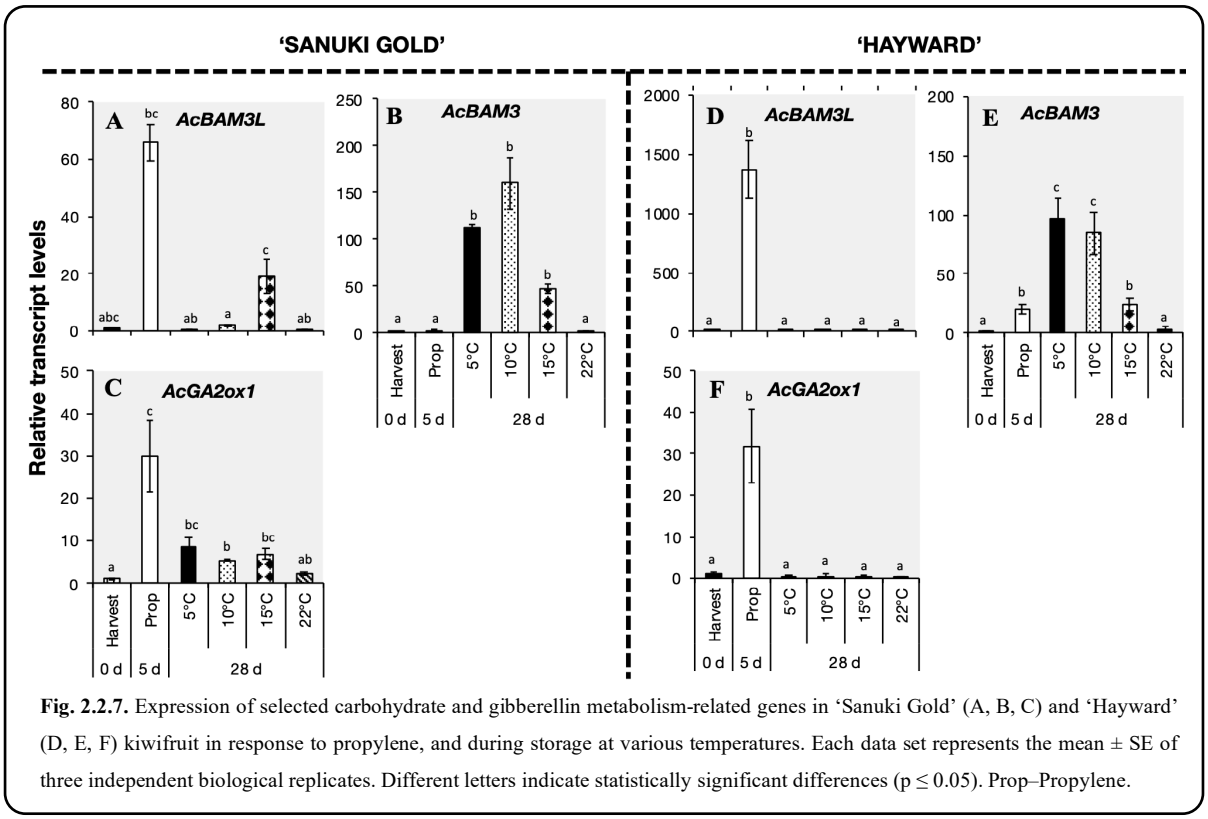


Fig. 2.2.7. Expression of selected carbohydrate and gibberellin metabolism-related genes in 'Sanuki Gold' (A, B, C) and 'Hayward' (D, E, F) kiwifruit in response to propylene, and during storage at various temperatures. Each data set represents the mean ± SE of three independent biological replicates. Different letters indicate statistically significant differences ($p \leq 0.05$). Prop–Propylene.

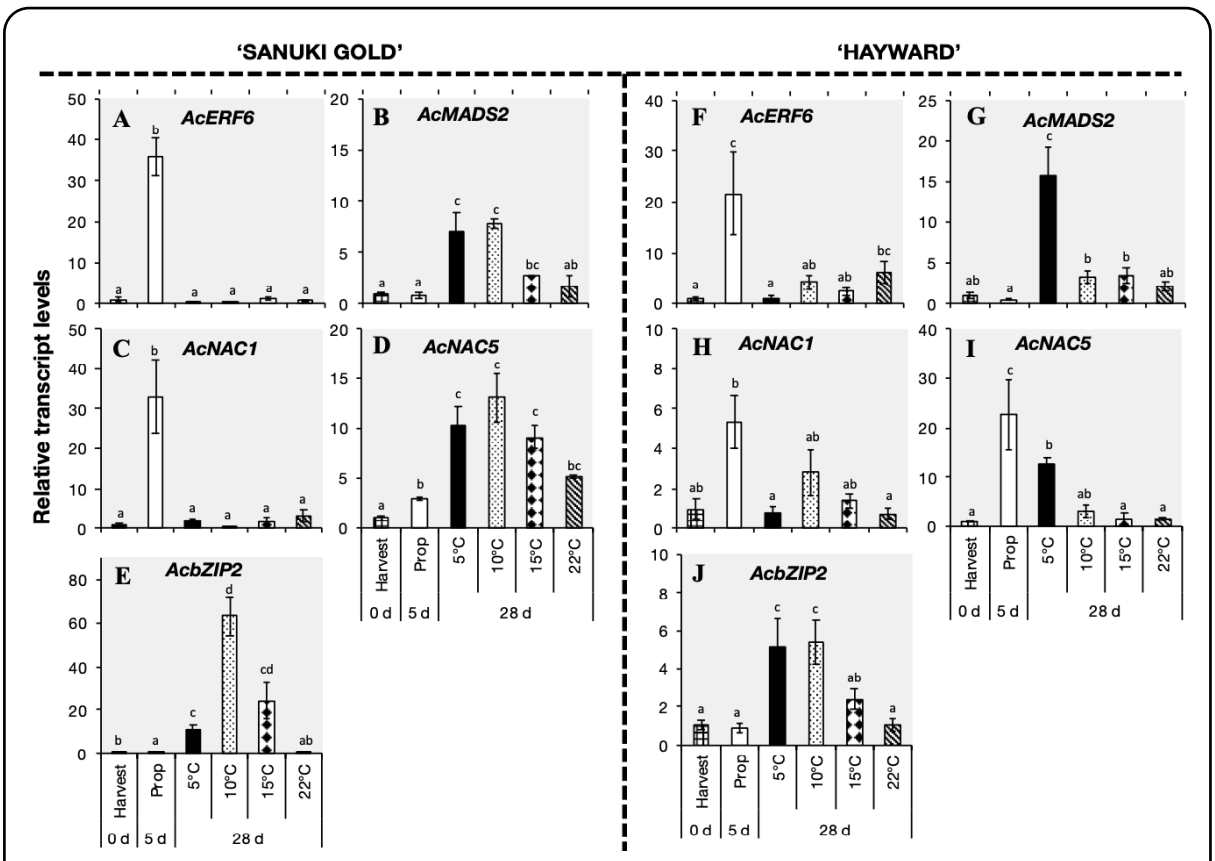


Fig. 2.2.8. Expression of selected genes encoding ripening–related transcription factors in 'Sanuki Gold' (A, B, C, D, E) and 'Hayward' (F, G, H, I, J) kiwifruit in response to propylene, and during storage at various temperatures. Each data set represents the mean ± SE of three independent biological replicates. Different letters indicate statistically significant differences ($p \leq 0.05$). Prop–Propylene.

2.2.4. Discussion

Fruit ripening comprises a set of processes that occur during the late stages of growth and development, and which are critical for improving the palatability of many fruit (Klee and Giovannoni, 2011). In climacteric fruit, such as kiwifruit, fruit ripening processes are largely driven by the phytohormone ethylene (Giovannoni, 2004; Klee and Giovannoni, 2011; Xu *et al*., 2012), which if left uncontrolled could cause excessive/premature ripening resulting in postharvest losses (Golden *et al*., 2014). However, kiwifruit are unlike other climacteric fruit since extensive ripening has been widely reported in the absence of any detectable ethylene (Hewett *et al*., 1999; Kim *et al*., 1999; Richardson *et al*., 2011; McAtee *et al*., 2015). The rate of ripening in kiwifruit during cold storage without detectable ethylene differs among kiwifruit cultivars with *Actinidia chinensis* var. chinensis fruit such as 'Sanuki Gold' softening faster within 1–2 months (Fukuda *et al*., 2007), unlike *Actinidia chinensis* var. deliciosa cultivars such as 'Hayward' which can be stored for up to 6 months (Arpaia *et al*., 1987; Ritenour *et al*., 1999). There is limited information on what regulates this peculiar ripening behaviour in kiwifruit. It has been demonstrated that low temperature can modulate fruit ripening independent of ethylene in several kiwifruit cultivars such as 'Sanuki Gold', 'Hayward' and 'Rainbow Red' (Mworia *et al*., 2012; Asiche *et al*., 2017; Asiche *et al*., 2018; Mitalo *et al*., 2018), but how ripening responses of different kiwifruit cultivars compare at various storage temperatures in the absence of detectable ethylene remain unclear. In this study, our main objective was to compare the ripening patterns and expression of related genes in two kiwifruit cultivars differing in maturity dates and postharvest storability at different storage temperatures under no detectable ethylene conditions.

Since kiwifruit are known to be sensitive to extremely low ethylene concentrations (Crisosto *et al*., 2000; Pranamornkith *et al*., 2012; Jabbar and East, 2016), it is important to eliminate any possible ethylene sources when conducting studies to unravel ethylene-independent ripening processes. It is well known that ethylene emanating from fruit infected with fungal pathogens such as *Botryosphaeria* sp., *Botrytis cinerea* and *Phomopsis* sp. pose a major problem to adjoining uninfected kiwifruit during storage (Koh *et al*., 2005; Manning *et al*., 2016). In this study, we constantly removed from the storage chambers, fruit that produced any detectable ethylene ($\geq$ 0.003 ng kg^{-1} s^{-1}), to avoid accumulation of the hormone during storage. These fruit later increased their ethylene production levels (Fig. 2.2.1), and developed rot symptoms within a few days after removal. The effect of disease-induced ethylene was alleviated by

storage at temperatures lower than 22 °C (Fig. 2.2.2), in agreement with previous reports (Koh *et al*., 2005).

Climacteric fruit ripening is often accompanied by a burst of ethylene production that is associated with changes in expression of biosynthesis genes *ACS* and *ACO* (Giovannoni, 2004; Klee and Giovannoni, 2011). In kiwifruit exposed to exogenous ethylene/propylene, the resulting ethylene burst is associated with increased expression of *ACS1* and *ACO2* (Richardson *et al*., 2011, McAtee *et al*., 2015). In both 'Sanuki Gold' and 'Hayward' kiwifruit, propylene treatment induced ethylene production (Appendix 1), which was strongly related to induction of *AcACS1* and *AcACO2* (Fig. 2.2.5A, B, E, F). However, neither *AcACS1* nor *AcACO2* were upregulated in kiwifruit during storage at all temperatures, accounting for the lack of detectable ethylene production. Instead, fruit during storage showed a significant change in expression of *AcACO3*, which was not affected at all by propylene (Fig. 2.2.5C, G), suggesting that its regulation was independent of ethylene. Ethylene-independent regulation of *AcACO3* by low temperature was also reported in our previous work (Asiche *et al*., 2018). In the present study, our results demonstrate that low temperature regulation of *AcACO3* is cultivar-dependent. While in 'Sanuki Gold' fruit, *AcACO3* was upregulated almost equally at 5 °C, 10 °C and 15 °C (Fig. 2.2.5C), its expression only increased substantially in 'Hayward' fruit at 5 °C and 10 °C (Fig. 2.2.5G). This suggests that 'Sanuki Gold' have a stronger response to low temperature than 'Hayward' fruit. This is further confirmed by the expression pattern of *AcETR2*, which was induced almost equally in 'Sanuki Gold' fruit at 5 °C, 10 °C and 15 °C (Fig. 2.2.5D), but only in 'Hayward' fruit at 5 °C and 10 °C (Fig. 2.2.5H).

Softening is one of the major indicators of fruit ripening in kiwifruit, and it is associated with many cell wall modification-related genes such as *PG* (Wang *et al*., 2000), *XET1* (Schröder *et al*., 1998), and *EXP1* and *PMEi* (Atkinson *et al*., 2011; Richardson *et al*., 2011; McAtee *et al*., 2015), whose regulation by ethylene has been previously established. In this study, softening in 'Sanuki Gold' fruit producing no detectable ethylene occurred almost at the same rate during storage at 5 °C, 10 °C and 15 °C to eating quality firmness within 28 d; fruit at 22 °C at the same timepoint were considerably firm (Fig. 2.2.3A, B). This faster softening of 'Sanuki Gold' fruit at 5 °C, 10 °C and 15 °C could be associated with increased expression of cell wall modification-related genes such as *AcXET2*, *AcPG*, *AcEXP1* and *AcPMEi* which were not induced at 22 °C (Fig. 2.2.6C, D, E, F). Faster softening and induction of related genes at 5 °C compared to 22 °C was also reported in our previous work on 'Sanuki Gold' kiwifruit (Mworia

et al., 2012; Asiche *et al*., 2018). However, the present results demonstrate that fruit softening and associated gene expression was equally induced at slightly higher temperatures (10 °C and 15 °C), suggesting a high sensitivity to low temperature in 'Sanuki Gold' fruit. Although significant softening was also observed in 'Hayward' fruit at 5 °C, 10 °C and 15 °C after 28 d, fruit at 5 °C were slightly softer than those at 10 °C and 15 °C (Fig. 2.2.3E, F). Analysis of the expression of cell wall modification-related genes revealed that while *AcPMEi* was induced at 5 °C, 10 °C and 15 °C (Fig. 2.2.6L), *AcPG* and *AcEXP1* were only significantly induced at 5 °C and 10 °C but not at 15 °C and 22 °C (Fig. 2.2.6J, K); *AcXET2* was not induced at all during storage of 'Hayward' fruit (Fig. 2.2.6I). The above results suggest that unlike 'Sanuki Gold' fruit, 'Hayward' fruit are less sensitive to low temperature stimulus and require much relatively lower temperatures (≤ 10 °C) to trigger the expression of cell wall modification-related genes and subsequent softening. Some cell wall modification genes such as *AcPL1* and *AcXET1* were exclusively induced by propylene in both kiwifruit cultivars (Fig. 2.2.6A, B, G, H); their lack of expression during storage indicates that the accelerated softening of 'Sanuki Gold' kiwifruit at 5 °C, 10 °C and 15 °C, and 'Hayward' fruit at 5 °C and 10 °C was independent of ethylene signalling. Moreover, frequent treatment of kiwifruit with 1-MCP, a potent ethylene perception inhibitor, failed to suppress the accelerated softening at lower storage temperatures (Fig. 2.2.4; Asiche *et al*., 2018), providing further evidence for the lack of ethylene signalling.

At the molecular level, fruit ripening is controlled by several transcription factors such as *MADS-RIN, FUL/TDR4, FUL2, TAGL1, NACs* and *bZIPs* that have been shown to bind and activate promoters of many ripening-associated genes (Vrebalov *et al*., 2002; Bemer *et al*., 2012; Lovisetto *et al*., 2013; Zhu *et al*., 2014; Nieuwenhuizen *et al*., 2015). In kiwifruit, autocatalytic ethylene production has been linked to the upregulation of *RIN/SEP4*, while *TDR4/FUL* was associated with ethylene-independent ripening regulation (McAtee *et al*., 2015). In this study, we have demonstrated that the induction of a new MADS-box related gene, *AcMADS2*, corresponds with the accelerated ripening and associated gene expression at low storage temperatures in kiwifruit, suggesting a mechanism by which softening-related genes were regulated (Fig. 2.2.8B, G). In 'Sanuki Gold' fruit, *AcMADS2* was induced during storage at both 5 °C and 10 °C, while it was only induced in 'Hayward' fruit at 5 °C, providing further evidence for differences in low temperature sensitivity between these two kiwifruit cultivars. This is further supported by the expression of *AcNAC5* and *AcbZIP2*, which were significantly induced in 'Sanuki Gold' fruit at 5 °C, 10 °C and 15 °C (Fig. 2.2.8D, E), but only in 'Hayward'

fruit at 5 °C and 10 °C (Fig. 2.2.8I, J). The expression of *AcMADS2* and *AcbZIP2* was not affected at all by propylene exposure in both cultivars (Fig. 2.2.8B, G, E, J), indicating that their regulation by low temperature was independent of ethylene. In addition, *AcERF6* and *AcNAC1* were induced by propylene exposure but not during storage (Fig. 2.2.8A, C, F, H), further suggesting that ethylene signalling is distinct from low temperature-induced ripening.

Another major indicator of fruit ripening in kiwifruit is the increase in SSC, which is associated with the degradation of starch by β-Amylases (Nardozza *et al*., 2013; Hu *et al*., 2016). In this study, SSC increase was reported for both 'Sanuki Gold' and 'Hayward' fruit at all storage temperatures (Fig. 2.2.3C, G), corresponding to increased expression of *AcBAM3* (Fig. 2.2.7B, E). Also, 1-MCP failed to suppress the SSC increase (Fig. 2.2.4), further suggesting that the changes were independent of ethylene. Ethylene-independent SSC increase was also reported in kiwifruit during storage at both low and room temperature (Arpaia *et al*., 1987; Boquete *et al*., 2004) and in 'Cantaloupe' melon (Flores *et al*., 2001). On the other hand, TA levels significantly decreased in a similar pattern as firmness, with faster rates of TA reduction at lower temperatures that is 5 °C, 10 °C and 15 °C for 'Sanuki Gold' fruit (Fig. 2.2.3D), and 5 °C and 10 °C for 'Hayward' fruit (Fig. 2.2.3H). Koukouranas and Sfakiotakis (2007) also reported a significant TA reduction in 'Hayward' kiwifruit during storage at 0 °C for eight weeks. Again, 1-MCP treatment failed to suppress the accelerated TA reduction at lower storage temperatures (Fig. 2.2.4), further supporting the hypothesis that low temperature induces ethylene-independent ripening in kiwifruit.

Consistent with previous findings (Atkinson *et al*., 2011), the expression of *AcGA2ox1* significantly increased in 'Sanuki Gold' fruit at 5 °C, 10 °C and 15 °C, but not in stored 'Hayward' fruit (Fig. 2.2.7C, F). While the actual role of *AcGA2ox1* in fruit ripening regulation is yet to be fully understood, its expression profile during storage provides a good indicator of differences in sensitivity to low temperature between 'Sanuki Gold' and 'Hayward' kiwifruit. Fruit ripening in kiwifruit attached to the vines also occurs in the absence of any detectable ethylene and is commonly referred to as Phase 1 ripening (Richardson *et al*., 2011; McAtee *et al*., 2015). Previously, we demonstrated that ethylene-independent ripening in on-vine 'Rainbow Red' kiwifruit coincided with the onset of winter and the accompanying gradual decrease in environmental temperature (Mitalo *et al*., 2018). These observations suggested that kiwifruit attached to the vines respond to changes in environmental temperature, and this could determine the onset of maturity and ripening. In Japan, 'Sanuki Gold' fruit are usually

harvested in early October, when the environmental temperatures drop to ~15 °C. This correlates with the observation in the present study that significant softening of 'Sanuki Gold' fruit occurred at 15 °C (Fig. 2.2.3A–D). By contrast, 'Hayward' fruit are late-maturing that are harvested in early November, when field temperatures drop to < 10 °C, also consistent with the findings of this study (Fig. 2.2.3E–H). Together, our results suggest that the differences in sensitivity to low temperature between 'Sanuki Gold' and 'Hayward' cultivars could also account for the disparities in their harvest maturity phenotypes.

2.2.5. Conclusion

Fruit ripening, depicted by a decrease in firmness, TA reduction and SSC increase, occurred in kiwifruit during storage in the absence of any detectable ethylene. Failure of 1-MCP to inhibit fruit ripening changes during kiwifruit storage indicated that they were independent of ethylene signalling. Comparisons of ripening responses between kiwifruit cultivars revealed that 'Sanuki Gold' fruit were more sensitive to low temperature, as storage at 15 °C was adequate to accelerate fruit softening and expression of associated genes such as *AcXET2*, *AcPG*, *AcEXP1* and *AcPMEi* within 28 d compared to 22 °C. This accelerated softening and expression of associated genes coincided with the induction of several genes encoding transcription factors such as *AcMADS2*, *AcNAC5* and *AcbZIP2*. On the other hand, 'Hayward' fruit exhibited a lesser sensitivity to low temperature as they required storage at temperatures ≤ 10 °C to accelerate softening and associated gene expression. This is further supported by the expression of other genes such as *AcACO3* and *AcGA2ox1* which were also significantly induced in 'Sanuki Gold' fruit even at 15 °C but only at ≤ 10 °C in 'Hayward' fruit. Therefore, the above differences in sensitivity to low temperature, between 'Sanuki Gold' and 'Hayward' kiwifruit, would account for the discrepancies observed in postharvest storability and harvest maturity phenotypes of these cultivars.

2.3. Determination of optimum temperature for long-term storage of kiwifruit

Abstract

Postharvest kiwifruit are largely susceptible to attack by pathogens, which induce ethylene production and premature ripening. While this effect is often reduced by cold storage, recent reports have demonstrated that relatively low storage temperatures (5–15 ºC) can accelerate kiwifruit ripening compared to room temperature storage. Therefore, a major challenge is to identify an optimum temperature that can circumvent the disastrous effects of both disease-induced ethylene and low temperature-modulated fruit ripening. In this study, 'Rainbow Red' kiwifruit harvested at a commercial maturity stage were stored at either 0 ºC, 2 ºC, 5 ºC or 22 ºC in ethylene-free chambers. Disease incidence (denoted by the percentage of ethylene-producing fruit) and changes in fruit firmness, soluble solids concentration (SSC) and titratable acidity (TA) were monitored at 4-week intervals during storage for up to 12 weeks. The expression of selected fruit ripening-associated genes was also examined using RT-qPCR. Results revealed that disease incidence was highest at 22 ºC; nevertheless, healthy kiwifruit maintained high firmness and TA for up to 8 weeks. By contrast, disease incidence was greatly reduced during storage at 5 ºC, and virtually absent at 0 ºC and 2 ºC. Fruit firmness and TA decreased gradually during storage at 2 ºC and 5 ºC, with fruit attaining eating quality within 8 weeks and 12 weeks, respectively. Storage at 0 ºC maintained high firmness and TA, and fruit at this temperature did not attain eating quality even after 12 weeks. The SSC increased during storage at all temperatures, although relatively lower levels were observed in fruit at 0 ºC. The expression levels of genes associated with cell wall modification (*AcPG*, *AcPL2*, *AcEXP1* and *AcXET2*), and carbohydrate metabolism (*Acβ-AMY1* and *Acβ-AMY2*) were substantially higher at low temperatures (0 ºC, 2 ºC and 5 ºC) than 22 ºC. Together, these observations suggested that 0 ºC is a suitable temperature for long-term storage of kiwifruit, while 2 ºC and 5 ºC are suitable for medium-term and short-term storage, respectively.

2.3.1. Introduction

Kiwifruit has grown and become an important commercial fruit in the world. From its original habitat in China, the growing area of kiwifruit has spread in New Zealand, Italy, Chile, United States, Spain, Korea and Japan among others (Park *et al*., 2006; Ferguson, 2014). The global export of kiwifruit has also increased tremendously. O'Rourke *et al*. (2012) reported that about

two-thirds of global kiwifruit is exported. Thus, to ensure a continuous supply of high-quality kiwifruit, it is necessary to extend their storage life.

The postharvest life of kiwifruit is largely influenced by the occurrence of fruit rots caused by *Botryoshaeria* spp., *Phomopsis* spp. And *Diaporthe actinidia* (Koh *et al*., 2005; Manning *et al*., 2016). Fruit infected by these diseases produce stress-induced ethylene, which results in premature softening of adjoining healthy kiwifruit. Kiwifruit is known to be very sensitive to exogenous ethylene, with concentrations as low as 0.01 μLL^{-1} adequately inducing ripening responses (Pranamornkith *et al*., 2012; Jabbar and East, 2016). Until now, low temperature and removal of ethylene remain the most effective tools for extending the storage life of kiwifruit.

'Rainbow Red' (*Actinidia chinensis*) is a commercially important early-season cultivar in Japan (Murakami *et al*., 2014). 'Rainbow Red' fruit are small (~100 g), have a green and smooth skin, and have yellow flesh with red coloration in the inner pericarp. The fruit have a sweet taste when ripe, with an average soluble solid concentration (SSC) of ~18 % and a total titratable acidity (TA) content of ~1 % making it a premium cultivar (Asiche *et al*., 2016; Murakami *et al*., 2014). However, 'Rainbow Red' kiwifruit have a major shortcoming as they can only be stored for 1−2 months at low temperature. Therefore, there is a need to develop new protocols to extend their storage life.

In our previous studies, we reported that low temperature can modulate fruit ripening independently of ethylene in various kiwifruit cultivars (Mworia *et al*., 2012, Asiche *et al*., 2017, Mitalo *et al*., 2018a, Mitalo et al., 2019a). We further revealed a myriad of kiwifruit ripening-associated genes that are regulated by low temperature in kiwifruit. Thus, finding a suitable storage temperature to prolong the postharvest life of kiwifruit is a fundamental question that remains unanswered. The purpose of the present study was to pinpoint the optimum storage temperature that can circumvent the occurrence of disease symptoms and low temperature-modulated ripening in order to maintain the postharvest quality of kiwifruit. Information from this study will be very useful in developing new and effective long-term storage protocols for kiwifruit.

2.3.2. Materials and methods

2.3.2.1. Plant material and treatments

'Rainbow Red' kiwifruit were harvested on 11th September 2015 from a commercial orchard in Takamatsu, Japan. Harvested kiwifruit were immediately transported to the Postharvest Horticulture Laboratory at Okayama University, Japan where they were sorted for uniform size, absence of defects, and lack of ethylene production. The fruit were then treated with a mixture of fungicides before being divided into four main groups. In each group, kiwifruit were individually wrapped in a perforated polythene bag and placed in a plastic container, separated from each other by ~10 cm. Fruit were stored in ethylene-free chambers set at either 0 ºC, 2 ºC, 5 ºC and 22 ºC for up to 12 weeks.

2.3.2.2. Determination of ethylene production and assessment of disease incidence

At each storage temperature, the ethylene production pattern of individual fruit was strictly monitored. Ethylene measurements were done by incubating fruit in a 440-mL container for 1 h and injecting 1 mL of headspace gas into a gas chromatograph (Model GC8 CMPF; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (set at 200 ºC) and an activated alumina column set at 80 ºC (Mworia *et al*., 2012). Fruit that produced detectable ethylene (≤ 0.01 $nLg^{-1}h^{-1}$) were removed from the storage chambers and monitored separately. These fruit showed increased ethylene production levels and developed senescent symptoms within a few days. Thus, disease incidence was determined as the percentage of ethylene producing fruit.

2.3.2.3. Assessment of fruit ripening characteristics

Fruit firmness, SSC and TA were determined using healthy fruit (five biological replicates) that did not produce any detectable ethylene. Sampling stages included fruit at harvest (day 0), and after storage for 4, 8 and 12 weeks. Fruit firmness was measured on two opposite cheeks of a sliced fruit using a penetrometer (model SMTT50; Toyo Baldwin, Tokyo, Japan) fitted with a 5-mm plunger (Mworia *et al*., 2012). SSC of the fruit juice was measured using a digital Atago PR1 refractometer (Atago Co. Ltd, Tokyo, Japan) and expressed as percentage. TA was determined by titrating the fruit juice against 0.1N NaOH using phenolphthalein as a pH indicator and was expressed as percentage citric acid equivalents. The outer pericarp of the fruit was then cut into small pieces and stored at −80 ºC for further analysis.

2.3.2.4. RNA extraction, cDNA synthesis and RT-qPCR analysis

Total RNA was extracted from fruit samples (three biological replicates) at day 0 and week 4 using a method for polysaccharide-rich fruit with slight modifications (Ikoma *et al*., 1996). The RNA was treated with Dnase I followed by clean-up using FavorPrep After TriReagent RNA Clean-up kit (Favorgen Biotech Co., PingTung, Taiwan). First strand cDNA was synthesized from 2.4 µg of RNA using RevTra Ace reverse transcriptase (Toyobo, Osaka, Japan) and random hexamer primer according to the manufacturer's instructions. Low temperature-responsive genes for RT-qPCR analysis were selected from our previous report (Asiche *et al*., 2018). Gene-specific primers (Table 2.3.1) were then designed using Primer3 software (version 0.4.0, http://bioinfo.ut.ee/primer3-0.4.0/). RT-qPCR analyses were conducted using MyiQ Single Colour Real-Time PCR detection system (BioRad, Hercules, CA) according to the manufacturer's instructions. The cycling condition was set at 95 ºC for 5 min followed by denaturation at 95 ºC for 5 s and annealing and extension at 59 ºC for 10 s (45 cycles). Relative gene expression was then calculated using *Actin* as the housekeeping gene, and day 0 samples set at 1.

Table 2.3.1. List of primer sequences used for RT-qPCR analysis in this study.

Gene	Gene ID	Name	Orientation	Sequence
AcPL2	HQ108112	Pectate lyase 2	Fw	AAGACGAGCCACTATGGATC
			Rv	CTGGCTTGCAATCGTGTATG
AcPG	AF152756	Polygalacturonase	Fw	TGGATTTGTTAGGGGTGTGC
			Rv	CAACTTGTGTCGCTGATGAC
AcEXP1	AY390358	Expansin 1	Fw	CGTGCTTCGAGCTAAAGTGC
			Rv	CGGCGATCTTGAGGAACATG
AcXET2	Achn387971	Xyloglucan endotransglycosylase 2	Fw	GCTTCTCACGCTTTCTCTCG
			Rv	GTTGACCCCAACGAAGACAG
AcßAMY1	FG525163	ß-Amylase 1	Fw	CCCCACATTGATGGAATGAC
			Rv	GTTTGTGATGCTGCCACTCG
AcßAMY2	Achn212571	ß-Amylase 2	Fw	CAGAGAACGCAAACTGCTCG
			Rv	GTTCCCGGAGTCTGATCTAC
AcACTIN	EF063572	Actin	Fw	TGGAATGGAAGCTGCAGGA
			Rv	CACCACTGAGCACAATGTTGC

2.3.2.5. Statistical analysis

Data from this study were subjected to ANOVA analysis with Duncan's new multiple range tests at $p \leq 0.05$ using the Agricolae package in R software version 3.3.2.

2.3.3. Results

2.3.3.1. Disease incidence during storage

Disease incidence was calculated as the percentage of fruit that produced detectable ethylene during storage. As shown in Fig. 2.3.1A, the proportion of kiwifruit that produced ethylene increased rapidly during storage at 22 ºC and by 8 weeks, the disease index was recorded at ~77 %. Consequently, kiwifruit storage tests at 22 ºC were terminated at this timepoint. During storage at 5 ºC, disease incidence was greatly reduced to just < 10 % after 8 weeks, but it sharply increased to 50 % after 12 weeks. By contrast, ethylene production and incidence of disease infection were virtually absent at 0 ºC and 2 ºC even after 12 weeks of storage.

2.3.3.2. Changes in kiwifruit ripening attributes

Fruit ripening attributes were determined using healthy kiwifruit that did not produce any detectable ethylene. Kiwifruit at harvest had a high firmness of ~54 N, low SSC of ~6 % and high TA of ~1.7 % (Fig. 2.3.1B–D). During storage, the fastest decrease in fruit firmness was observed at 5 ºC, reaching ~10 N after 4 weeks, ~4 N after 8 weeks and ~1 N after 12 weeks (Fig. 2.3.1B). Fruit at 2 ºC also exhibited a decrease in firmness but a relatively slower rate than those at 5 ºC; ~44 N after 4 weeks, ~11 N after 8 weeks, and ~1 N at 12 weeks. On the other hand, there was a very slow rate of firmness reduction during storage at 22 ºC whereby fruit maintained high flesh firmness (~30 N) after 8 weeks. Fruit stored at 0 ºC also maintained a high flesh firmness (~27 N) for 12 weeks. Kiwifruit SSC increased gradually to > 15 % during storage at all temperatures, although the levels were generally lower in fruit at 0 ºC (Fig. 2.3.1C). A decrease in fruit TA was also observed at all temperatures with fruit at 5 ºC depicting the fastest rate of decrease while fruit at 0 ºC and 22 ºC maintain high TA levels (Fig. 2.3.1D).

2.3.3.3. Expression of fruit ripening-associated genes

The expression of six selected kiwifruit ripening-associated genes was analysed by RT-qPCR in fruit collected at harvest and after storage at 0 ºC, 2 ºC, 5 ºC and 22 ºC for 4 weeks (Fig. 2.3.2). These genes were previously shown to be inducible by low temperature in our previous

studies (Asiche *et al*., 2018; Mitalo *et al*., 2018a; Mitalo *et al*., 2019a). The expression of *AcPG* was highest in fruit at 5 ºC with a 450-fold increase compared to 2 ºC (45-fold) and 0 ºC (26-fold); *AcPG* expression changes at 22 ºC were very minimal (7-fold) (Fig. 2.3.2A). *AcPL2* expression increased faster during storage at 5 ºC (340-fold) and 2 ºC (215-fold) compared to 22 ºC (75-fold) (Fig. 2.3.2B). No significant changes in the expression of *AcPL2* were recorded at 0 ºC. The expression of *AcXET2* also showed the highest increase during storage at 5 ºC (280-fold), with only 160-fold and 95-fold increases at 2 ºC and 0 ºC respectively (Fig. 2.3.2C). *AcXET2* expression changed only slightly (7-fold) during storage at 22 ºC. The other softening-related gene, *AcEXP1*, exhibited a substantial expression increase only in fruit at 5 ºC (9-fold), while there were no significant changes during storage at 0 ºC, 2 ºC and 22 ºC (Fig. 2.3.2D).

The two carbohydrate metabolism genes that were analysed showed contrasting expression patterns. The expression of *Acβ-AMY1* increased markedly during storage at 5 ºC (490-fold) and 2 ºC (220-fold), while the expression changes were relatively lower at 0 ºC (20-fold) and 22 ºC (60-fold) (Fig. 2.3.2E). Contrarily, *Acβ-AMY2* expression increase was higher at 0 ºC (510-fold) than at 2 ºC (200-fold), 5 ºC (100-fold) and 22 ºC (26-fold) (Fig. 2.3.2F).

2.3.4. Discussion

Long-term storage is one of the major goals of postharvest handling of horticultural produce towards extending the period of availability, allow for transportation of fruit to distant markets, and preservation of nutritional qualities (Wills and Golding, 2016). Kiwifruit are largely affected by postharvest pathogens including *Botryosphaeria* sp., *Botrytis* sp. and *Phomopsis* sp. That pose a major problem during postharvest handling (Koh *et al*., 2005; Manning *et al*., 2016). Disease-induced ethylene emanating from infected fruit is a leading cause of premature ripening during storage (Manning *et al*., 2016; Asiche *et al*., 2018). In this study, low storage temperatures dramatically reduced the incidence of disease infection and proportion of ethylene-producing fruit (2.3.1A), which is in close agreement with previous findings (Manning *et al*., 2016). Interestingly, the suppression of disease incidence at 5 ºC was limited to only 8 weeks, while storage at 0 ºC and 2 ºC effectively suppressed disease incidence for up to 12 weeks. These results suggested that 0 ºC and 2 ºC would be very effective temperatures for long-term storage of kiwifruit.

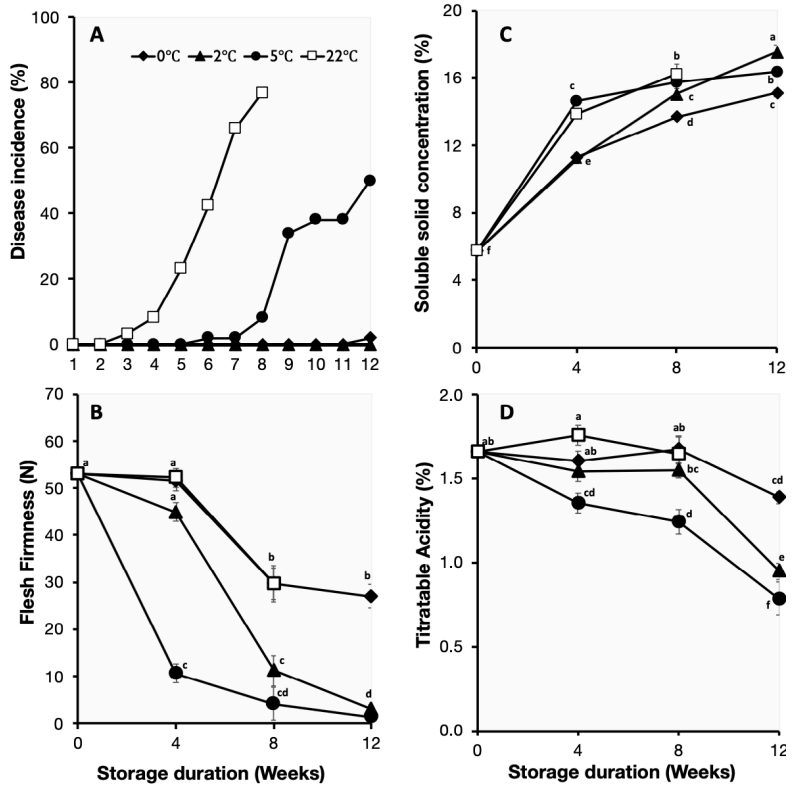


Fig. 2.3.1. 'Rainbow Red' kiwifruit ripening attributes during storage at different temperatures. **A.** Disease incidence; calculated as percentage of fruit producing detectable ethylene. **B.** Fruit firmness, **C.** Soluble solids concentration, and **D.** Titratable acidity were determined using healthy fruit that did not produce any detectable ethylene. Different letters indicate significant difference at $p \leq 0.05$.

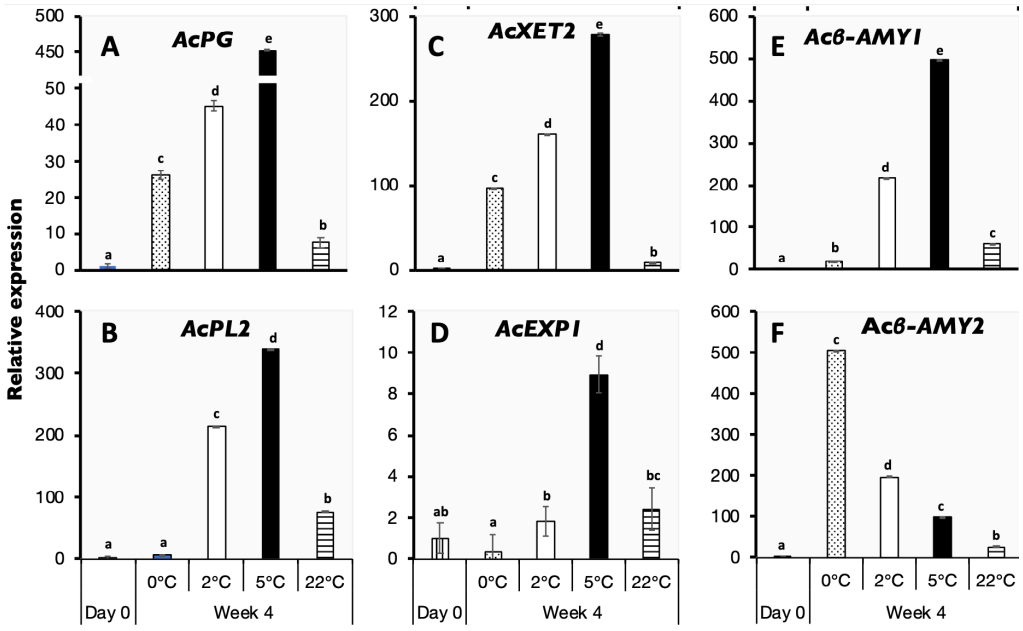


Fig. 2.3.2. Expression of selected kiwifruit ripening-associated genes during storage at various temperatures. Relative expression was determined by RT-qPCR analysis using gene-specific primers for **A.** *AcPG* (*Polygalacturonase*, Achn051381), **B.** *AcPL2* (*Pectate Lyase 2*, Achn315151), **C.** *AcXET2* (*Xyloglucan Endotransglycosylase*, Achn023381), **D.** *AcEXP1* (*Expansin 1*, Achn336951), **E.** *Acβ-AMY1* (*β-Amylase 1*, Achn141771) and **F.** *Acβ-AMY2* (*β-Amylase 2*, Achn212571). Different letters indicate significant difference at $p \leq 0.05$.

During storage, fruit ripening changes, especially firmness and TA reduction, were accelerated at lower temperatures (0 ºC, 2 ºC and 5 ºC) than 22 ºC in the absence of detectable ethylene (Fig. 2.3.1B–D). This is consistent with previous findings that low temperature modulates kiwifruit ripening independently of ethylene (Mworia *et al*., 2012; Asiche *et al*., 2017; Asiche *et al*., 2018; Mitalo *et al*., 2018a; Mitalo *et al*., 2019a). Kiwifruit softening was also reported to be accelerated by low storage temperature in other independent studies (Hewett *et al*., 1999; Kim *et al*., 1999, Jabbar and East, 2016). Among the low storage temperatures, the fastest reduction in firmness and TA was recorded at 5 ºC whereby fruit attained eating quality ripeness stage within 4–8 weeks. Fruit softening and TA reduction also occurred relatively faster at 2 ºC to eating quality ripeness stage after 8–12 weeks. It is however intriguing that fruit at 0 ºC maintained high firmness and TA for up to 12 weeks. These findings further support the potentiality of 0 ºC as an optimum temperature for long-term storage.

It widely recognized that fruit ripening is transcriptionally regulated by the upregulation and downregulation of numerous genes (Bouzayen *et al*., 2010). Fruit softening in kiwifruit is linked to upregulation of several genes associated with cell wall modification such as *AcPG*, *AcXET*, *AcEXP*, *AcPL2*, and *Acβ-GAL* among others (Schröder and Atkinson, 2006; Yang *et al.,* 2007; Atkinson *et al*., 2009; Mworia *et al*., 2012). In this study, the rapid fruit softening at 5 ºC would be caused by high expression levels of *AcPG*, *AcPL2*, *AcXET2* and *AcEXP1* (Fig. 2.3.2A–D). Equally, fruit softening changes observed at 2 ºC could be linked to increased expression of *AcPG*, *AcPL2* and *AcXET2* (Fig. 2.3.2A–C). On the other hand, the high firmness values of fruit at 22 ºC could be attributed to minimal changes in expression levels of the cell wall modification-associated genes. It is incredible that *AcPG* and *AcXET2* expression levels increased substantially during storage at 0 ºC whereas fruit maintained high firmness (Fig. 2.3.2A, C). This discrepancy could be due to low protein accumulation and/or enzyme activity that would rather slow down the fruit softening rate.

Fruit SSC increased during storage at all temperatures (Fig. 2.3.1C), and this could be attributed to an appreciable increase in expression levels of *Acβ-AMY1* and *Acβ-AMY2* at 0 ºC, 2 ºC, 5 ºC and 22 ºC (Fig. 2.3.2E, F). Previous studies have also linked several *β-AMYs* to starch degradation and soluble sugar accumulation during kiwifruit ripening (Nardozza *et al*., 2013; Hu *et al*., 2016). In the present study, low expression levels of *Acβ-AMY1* and *Acβ-AMY2* at 22 ºC could be compensated by higher protein accumulation and/or metabolic rates, which would account for the similar SSC levels during storage.

2.3.5. Conclusion

The results presented in this study demonstrated that kiwifruit ripening was accelerated by low temperature in the absence of any detectable ethylene. Although healthy fruit at room temperature (22 ºC) maintained a high firmness, the incidence of ethylene production (due to disease infections) was extremely high. This finding rules out the possibility of using room temperature for long-term storage. Storage at 2 ºC and 5 ºC considerably reduced disease incidence and ethylene production. However, fruit ripening and associated gene expression were induced after 4 weeks at 5 ºC and 8 weeks at 2 ºC. This implies that 5 ºC might only be suitable for short-term storage for about 4 weeks while 2 ºC was suitable for medium-term storage for about 8 weeks. Disease incidence and fruit ripening changes were effectively suppressed at 0 ºC, demonstrating the potentiality of this temperature for long-term storage of kiwifruit for over 12 weeks.

2.4. Soluble sugar and aroma volatile changes during ethylene-dependent and low temperature-modulated fruit ripening in kiwifruit

Abstract

Fruit ripening in response to propylene (an ethylene analogue), 1-methylcyclopropene (1-MCP, an ethylene action inhibitor), and low temperature (5 ºC) treatments was characterized in 'Kosui' kiwifruit (*Actinidia rufa* × *A. chinensis*). Propylene treatment induced ethylene production, with increased expression levels of *1-aminocyclopropane-1-carboxylic acid* (*ACC*) *synthase 1* (*AcACS1*) and *ACC oxidase 2* (*AcACO2*), and rapid fruit softening together with increased expression levels of *polygalacturonase* (*AcPG*) and *expansin 1* (*AcEXP1*) within 5 d. Fruit soluble solids concentration (SSC) and contents of sucrose, glucose, and fructose together with the expression levels of *β-amylase 1* (*Acβ-AMY1*), *Acβ-AMY2*, and *invertase* (*AcINV3-1*) increased rapidly after 5 d exposure to propylene. Furthermore, propylene exposure for 5 d was sufficient to induce the production of key aroma volatile compounds, ethyl- and methyl butanoate, accompanied with increased expression levels of *alcohol acyl transferase* (*AcAAT*). Application of 1-MCP at the start of the experiment, followed by continuous exposure to propylene, significantly delayed fruit softening, changes in SSC and sugars, and strongly suppressed the production of ethylene, aroma volatiles, and expression of associated genes. During storage, fruit softening, SSC and sugar increase, and increased expression of genes associated with cell wall modification and carbohydrate metabolism were registered without detectable ethylene production; however, these changes occurred faster at 5 ºC compared to 22 ºC. Interestingly, ethyl and methyl butanoate as well as *AcAAT* expression were undetectable in kiwifruit during storage, while they were rescued by post-storage propylene exposure, indicating that the production of aroma volatile compounds is strongly ethylene-dependent. Transcript levels of a NAC-related transcription factor (TF), *AcNAC3*, increased in response to both propylene and low temperature treatments, while *AcNAC5* was exclusively up-regulated by propylene. By contrast, transcript levels of a MADS-box TF, *AcMADS2*, exclusively increased in response to low temperature. The above findings indicate that kiwifruit ripening is inducible by either ethylene or low temperature signals. However, fruit ripened by low temperature were deficient in ethylene-dependent aroma volatiles, suggesting that ethylene signalling is non-functional during low temperature-modulated ripening in kiwifruit. These data provide further evidence that ethylene-dependent and low temperature-modulated ripening in kiwifruit involve different regulatory mechanisms.

2.4.1. Introduction

The phytohormone ethylene regulates a wide range of plant growth and developmental processes, including fleshy fruit ripening (Lashbrook *et al*., 1998; Giovannoni, 2004). During ripening, fleshy fruit undergo various physiological, biochemical, and structural changes including softening, starch degradation to sugars, pigment accumulation, and production of aroma volatiles (Klee and Giovannoni, 2011; Osorio *et al*., 2013). The onset of fruit ripening in climacteric fruit such as tomatoes, apples, and peaches is accompanied by a marked increase in ethylene production (Xu *et al*., 2012), which is regulated at the transcriptional level via the differential expression of genes encoding two key enzymes: 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) (Wang *et al*., 2002; Cherian *et al*., 2014).

Kiwifruit (*Actinidia* spp.) are categorized as climacteric, since fruit ripening is largely driven by ethylene-regulated changes in gene expression (Antunes, 2007; Yin *et al*., 2008). Exogenous ethylene or propylene treatment initiates rapid fruit softening through the induction of several cell wall modification-associated genes such as *polygalacturonase* (*AcPG*) and *expansin 1* (*AcEXP1*) (Wang *et al*., 2000; Atkinson *et al*., 2011; Mworia *et al*., 2012). In addition, kiwifruit respond to exogenous ethylene/propylene by increasing their soluble solids concentration (SSC), which is associated with the induction of various starch degradation-related genes such as *β-amylase* (*Acβ-AMY*) (Nardozza *et al*., 2013; McAtee *et al*., 2015; Hu *et al*., 2016). Ripe kiwifruit produces a composite of aroma volatiles, consisting of mainly aldehydes and esters (Marsh *et al*., 2006). Characteristic kiwifruit esters have been identified as ethyl butanoate and methyl butanoate (Zhang *et al*., 2009), and their regulation by ethylene has been previously described (Atkinson *et al*., 2011; Günther *et al*., 2011; Günther *et al*., 2015). Ethylene-induced ripening changes in kiwifruit are usually followed by a sharp increase in ethylene production caused by the up-regulation of key biosynthetic genes *AcACS1* and *AcACO2* (Pratt and Reid, 1974; Mworia *et al*., 2010; Atkinson *et al*., 2011; McAtee *et al*., 2015).

Kiwifruit exhibit a peculiar ripening behaviour, as substantial softening in healthy intact fruit occurs during low temperature storage in the absence of any detectable ethylene (Antunes, 2007; Yin *et al*., 2009). Arpaia *et al*. (1987) demonstrated that kiwifruit are sensitive to

extremely low ethylene concentrations (as low as 0.01 μLL^{-1}). Consequently, the substantial softening during storage at < 1.5 ºC in air (presumed to contain 0.001 μLL^{-1} ethylene) is believed to be controlled by basal levels of system I ethylene that is present in most fruit and plant tissues (Kim *et al*., 1999; Pranamornkith *et al*., 2012; Jabbar and East, 2016). However, to date, there is no direct evidence linking system I ethylene to kiwifruit ripening during low temperature storage, and thus, the regulatory mechanisms associated with this phenomenon remain less well understood.

Over the past few years, this study group has been dedicated to elucidating the molecular mechanisms underpinning low temperature-modulated fruit ripening in kiwifruit. A preliminary study by Mworia *et al*. (2012) demonstrated that healthy intact 'Sanuki Gold' kiwifruit softened; accumulated *AcPG*, *pectate lyase* (*AcPL*), and *AcEXP1* mRNAs; and decreased their titratable acidity (TA) during storage at 4 ºC, but not at 25 ºC, despite the lack of any detectable increase in ethylene production. These changes were not suppressed by frequent application of 1-methylcyclopropene (1-MCP) to keep fruit insensitive to ethylene, indicating that they occur outside the sphere of ethylene influence. Similar results were reported using different kiwifruit cultivars (Asiche *et al*., 2017; Mitalo *et al*., 2018a; Mitalo *et al*., 2019a), confirming that low temperature-modulated ripening is common to all kiwifruit cultivars. Follow-up transcriptomic studies revealed that a distinct set of ripening-associated genes in kiwifruit were uniquely regulated by low temperature, independent of ethylene (Asiche *et al*., 2018). Despite these findings, low temperature-modulated ripening in kiwifruit remains a poorly understood phenomenon. There is a growing need to conduct more focused studies to examine the similarities and differences in the molecular regulation of ethylene-induced and low temperature-modulated ripening in kiwifruit.

Using gas chromatography-mass spectrometry (GC-MS) to analyse the volatiles and soluble sugar contents of 'Kosui' kiwifruit, an interspecific hybrid between *Actinidia rufa* and *A. chinensis*, this study sought to investigate the impact of low temperature (5 ºC) on sugar and aroma volatile profiles relative to ethylene effect. Changes in sugar composition and aroma volatile production in response to propylene, 1-MCP, and during storage at 5 ºC and 22 ºC were compared, and their concomitant gene expression patterns are reported. Our results indicate that the production of aroma volatiles is strongly ethylene-dependent and is absent during cold storage, providing evidence that ethylene signalling is non-functional during low temperature-modulated ripening.

2.4.2. Materials and methods

2.4.2.1. Plant material and treatments

'Kosui' kiwifruit grown under standard cultural practices were harvested from a commercial orchard in Takamatsu, Japan at a physiological maturity stage [170 days (d) after full bloom (DAFB), firmness: 73.02 ± 3.39 N, SSC: 7.79 ± 0.18 %, TA: 2.20 ± 0.03 %]. After harvesting, careful sorting was conducted to exclude fruit with physical injuries, disease symptoms, and those producing ethylene. Fruit were then divided into five sets, corresponding to the various treatments.

2.4.2.2. Ethylene-dependent fruit ripening

Three sets of 30 fruit each were used in this experiment. The first set was held in gas-tight plastic containers that were continuously treated with 5000 μLL^{-1} propylene, a well-known ethylene analogue (McMurchie *et al*., 1972; Mworia *et al*., 2010; Asiche *et al*., 2016). Propylene treatment was done to induce ethylene signalling, and to allow for determination of endogenous ethylene produced by the fruit. The second set was initially exposed to 2 μLL^{-1} 1-MCP for 12 h followed by continuous exposure to 5000 μLL^{-1} propylene. 1-MCP was released by dissolving SmartFresh™ powder (AgroFresh, PA, United States) in water. The third set contained non-treated fruit as a control. All treatments were carried out at 22 ºC for up to 9 d. Soda lime was placed in plastic containers during propylene and 1-MCP treatments to reduce CO_2 accumulation.

2.4.2.3. Low temperature-modulated fruit ripening

Two sets of 300 fruit each were stored either at 5 ºC or 22 ºC in air for up to 49 d. During storage, fruit were individually wrapped in perforated polythene bags to reduce water loss, before placing them (~10 cm apart) in plastic trays. Ethylene production pattern of each fruit was monitored at weekly intervals throughout the storage period. To avoid ethylene accumulation in the storage chambers, fruit that produced detectable ethylene (< 10 %) were excluded based on previous observations that ethylene production correlated with the appearance of disease symptoms (Asiche *et al*., 2018). At the end of the storage period, fruit at 5 ºC were further divided into two groups before being transferred to 22 ºC for up to 14 d; one group was continuously treated with propylene as described above in section 2.4.2.2, while the

other group was left untreated. Similarly, fruit at 22 ºC were divided into two groups; one group was continuously treated with propylene and the other one was left untreated.

2.4.2.4. Evaluation of changes in ethylene production, firmness, SSC, and TA

To determine ethylene production, individual fruit were incubated in a 440-mL container for up to 1 h. Ethylene production rate was measured by withdrawing 1 mL of headspace gas and injecting it into a gas chromatograph (Model GC4 CMPF, Shimadzu, Kyoto, Japan), equipped with a flame ionization detector (set at 200 ºC) and an activated alumina column (set at 80 ºC) (Mworia *et al*., 2012). This procedure has a minimum ethylene detection capacity of 0.01 $nLg^{-1}h^{-1}$. Fruit firmness was measured at two equatorial regions of peeled fruit using a penetrometer (model SMT-T-50, Toyo Baldwin, Tokyo, Japan) fitted with an 8-mm plunger. Data were recorded as Newtons (N) and firmness was expressed as a mean of five independent biological replications. SSC and TA were determined using fruit juice as described elsewhere (Asiche *et al*., 2018; Mitalo *et al*., 2019a).

2.4.2.5. Collection of aroma volatiles

Aroma volatile compounds were collected according to the procedure by Sobhy *et al*. (2017), with slight modifications. Intact fruit were placed into sealed 1360-mL containers equipped with two ports; one for the air inlet and another port for the outlet. Dried air, purified by passing through a charcoal filter, was introduced to sweep through the headspace at approximately 0.75 L min^{-1}. The air was then pulled out through the outlet port fitted with a custom-made 10-cm glass trap (5 mm inner diameter), filled with Porapak Q (200 mg, Supelco Analytical, Bellefonte, PA, United States) held in place by two plugs of silanized glass wool. Porapak Q traps were conditioned before use by flushing with 2 mL methanol (Sigma-Aldrich Co., United States), followed by 2 mL dichloromethane (DCM, Wako Pure Chemical Industries, Japan), dried, and then placed overnight in an oven at 60 ºC for complete drying. To exclude the effect of background volatiles in air, empty containers having no fruit were included in the collection setup. Aroma volatiles were collected over 24 h periods. After each collection period, volatile compounds were eluted from Porapak Q traps with 1 mL DCM after adding 400 ng tetralin (1,2,3,4-tetrahydronaphthalene; Nacalai Tesque, Japan) on the top of each column. Tetralin was used as an internal standard. The samples were stored in 1.5 mL glass vials at -20 ºC until further analysis.

2.4.2.6. Extraction and derivatization of soluble sugars

Soluble sugars were obtained and derivatized as described by Wang *et al*. (2010). Briefly, the samples (0.1 g) were ground in liquid nitrogen and extracted in 1.4 mL of 100 % methanol with ribitol (12 µg) added as an internal standard. After fractionating the non-polar metabolites into chloroform, 150 µL of the polar phase was transferred into a 1.5 mL micro-centrifuge tube to measure the metabolites (soluble sugars). These were dried under vacuum without heating, flushed with nitrogen gas, and then derivatized sequentially with methoxyamine hydrochloride and N-methyl-N-trimethylsilyl-trifluoroacetamide (Lisec *et al*., 2006). The samples were stored at -20 ºC until further analysis.

2.4.2.7. GC-MS conditions and chemical analysis

Volatile eluates and metabolites were analysed using an Agilent 240 GC-MS ion trap system coupled with Agilent 7891A GC fitted with the HP-5MS column (5 % phenyl methyl silox, 30 m length × 0.25 mm inner diameter × 0.25 µm film thickness) (Agilent Technologies, Santa Clara, CA, United States). One microliter of each eluted sample was injected in split mode (1:30) into the injector port of the GC instrument held at 230 ºC via an Agilent 7693A auto-sampler. Helium (1 mL/min) was used as carrier gas, and MS ionization was achieved by electron impact (EI) at emission current 30 µAmps for volatiles (10 µAmps for metabolites) in the ion trap held at 200 ºC (transfer line was 260 ºC). For headspace volatile analyses, the GC oven temperature was programmed at 40 ºC for 3 min, and then increased at 5 ºC min^{-1} to 180 ºC, followed by 20 ºC min^{-1} ramp to 300 ºC, where it was held for an additional 5 min before returning to initial conditions. For metabolite samples, GC temperature program was 5 min at 60 ºC, followed by 5 ºC min^{-1} to 300 ºC, 5 min hold period, and return to initial temperature and equilibration. MS data were collected in full scan mode in mass range *m/z* 40–300 for volatiles (*m/z* 40–750 for metabolites) and analysed by Agilent Workstation Version 7.0.2 software. Aroma volatiles and metabolites were identified by comparing their fragmentation patterns with those from the NIST 2011 Mass Spectral Library and Software (National Institute of Standards and Technology, United States). Co-injection with authentic standards was undertaken to confirm tentative identifications where possible. Quantifications were based on standard curves generated for each target compound and internal standards.

2.4.2.8. RNA extraction

Total RNA was extracted from ~3 g of outer pericarp tissue (three biological replications) using a method for polysaccharide-rich tissues (Ikoma *et al*., 1996), with slight modifications. DNase I (Nippon Gene, Tokyo) treatment followed by clean-up using FavorPrep after Tri-Reagent RNA Clean-up Kit (Favorgen Biotech Co., Pingtung, Taiwan) were carried out to remove genomic DNA contamination from the extracted RNA.

2.4.2.9. Quantitative Real-Time PCR (RT-qPCR)

The method used was similar to that reported in our previous study (Asiche *et al*., 2018). Briefly, first strand cDNA was synthesized from 2.4 µg of RNA using RevTra Ace reverse transcriptase (Toyobo, Osaka, Japan), and random hexamer primer according to the manufacturer's instructions. Gene-specific primers (Table 2.4.1) for RT-qPCR analysis were designed using Primer3 software (version 0.4.0; http://bioinfo.ut.ee/primer3-0.4.0/). Gene expression of three biological replications was examined on a MyiQ Single-Color Reverse Transcriptase-Quantitative PCR Detection System (Bio-Rad, Hercules, CA, United States) using TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. *AcActin* was used as the housekeeping gene. The specificity of all primers was verified by melting curve analysis. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method with samples at harvest (0 d) calibrated as 1.

2.4.2.10. Statistical analysis

Data obtained in this study were subjected to statistical analyses using R software (version 3.4.0, R Project). ANOVA followed by post hoc Tukey's tests ($p \leq 0.05$) were used to detect differences in fruit ripening characteristics and gene expression levels among the different treatments.

Table 2.4.1. Oligonucleotide sequences of primers used for RT-qPCR analysis in this study.

Name	Gene ID	Description		Sequence (5' to 3')	Reference
AcACS1	Achn364251	1-Aminocyclopropane-1-carboxylic acid synthase	5' to 3'	GAAAGGCTGCGTGCAATTCTC	Asiche et al. (2018)
			3' to 5'	CCTGAAAATGGACTGCCCATC	
AcACO2	Achn326461	1-Aminocyclopropane-1-carboxylic acid oxidase	5' to 3'	TCTCAGAAATCCCCGATCTCG	Asiche et al. (2018)
			3' to 5'	TTGGAGCCACTGAAAGCCTTC	
AcPG	Achn051381	Polygalacturonase	5' to 3'	TGGATTTGTTAGGGGTGTGC	Asiche et al. (2018)
			3' to 5'	CAACTTGTGTCGCTGATGAC	
AcEXP1	Achn336951	Expansin	5' to 3'	CGTGCTTCGAGCTAAAGTGC	Asiche et al. (2018)
			3' to 5'	CGGCGATCTTGAGGAACATG	
Acβ-AMY1	Achn141771	β-Amylase 1	5' to 3'	CCCCACATTGATGGAATGAC	Asiche et al. (2018)
			3' to 5'	GTTTGTGATGCTGCCACTCG	
Acβ-AMY2	Achn212571	β-Amylase 2	5' to 3'	CAGAGAACGCAAACTGCTCG	Asiche et al. (2018)
			3' to 5'	GTTCCCGGAGTCTGATCTAC	
AcINV3-1	Achn319711	Vacuolar invertase	5' to 3'	CATAGTCCTTGCCGACCATT	Nardozza et al. (2013)
			3' to 5'	GAGCGAAGCTCTCCACAATC	
AcAAT	KJ626345	Alcohol acyl transferase	5' to 3'	GAAATGTCTTTGCCCTTCCG	Asiche et al. (2018)
			3' to 5'	CCCCACCCAAAATCTATCGC	
AcNAC3	Achn134171	NAC transcription factor 3	5' to 3'	ATTGACAAGCCGGTGCTCA	Asiche et al. (2018)
			3' to 5'	TGGCTTGGATTTCGCTCTG	
AcNAC5	Achn169421	NAC transcription factor 5	5' to 3'	GAATTGCCCGAGAAAGCAGA	Asiche et al. (2018)
			3' to 5'	TGTCTTGATACCCTTCGGTGG	
AcMADS2	Achn235371	MADS-box transcription factor	5' to 3'	GGACAAGAACAGTCGCCAGG	Mitalo et al. (2018)
			3' to 5'	GTATCTGTCGCCGGTGATG	
AcACTIN	EF063572	Actin; housekeeping gene	5' to 3'	TGGAATGGAAGCTGCAGGA	Asiche et al. (2018)
			3' to 5'	CACCACTGAGCACAATGTTGC	

2.4.3. Results

2.4.3.1. Induction of ethylene biosynthesis in postharvest kiwifruit

At harvest, kiwifruit used in this study did not produce any detectable ethylene levels. To understand the molecular mechanisms responsible for ethylene production during postharvest handling, we determined the effects of propylene and 1-MCP treatments, as well as storage temperature (Fig. 2.4.1).

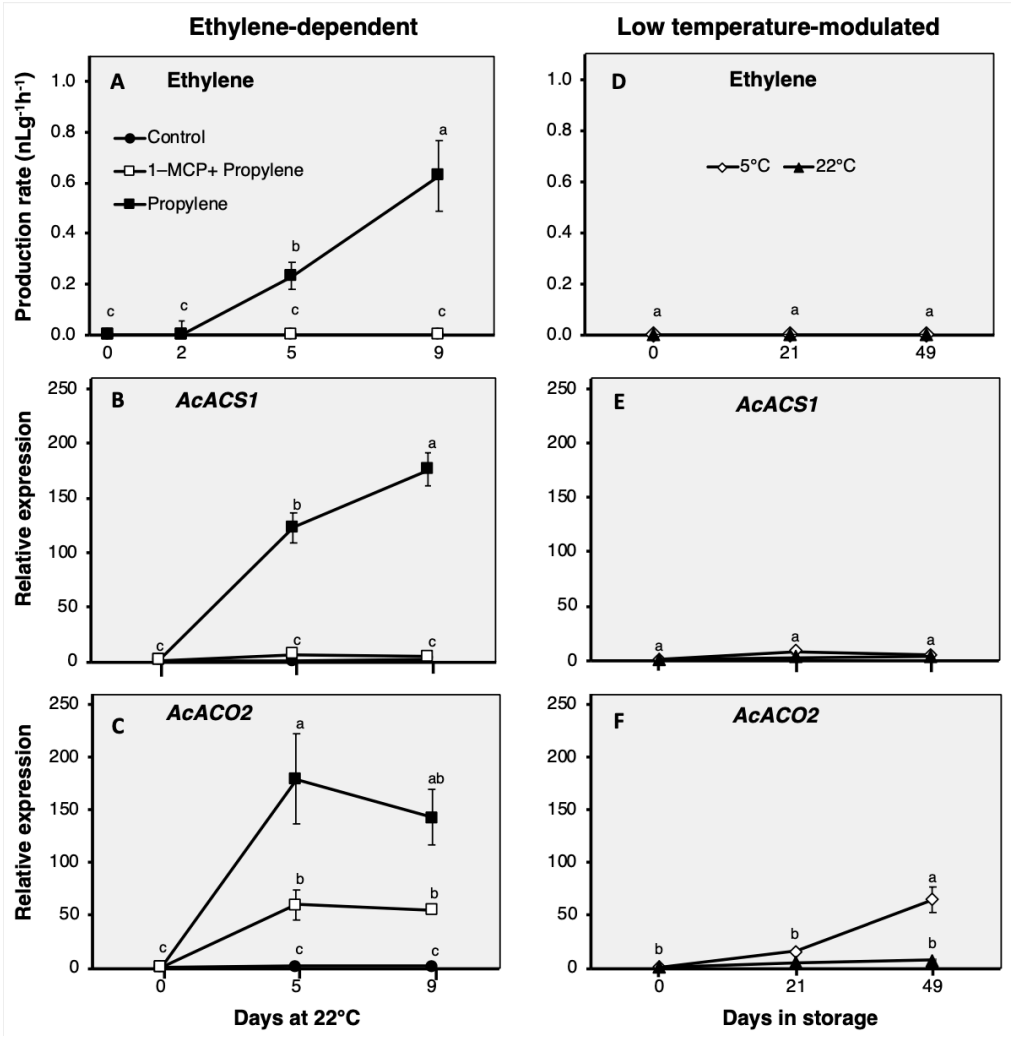


Fig. 2.4.1. Ethylene production and expression patterns of ethylene biosynthetic genes in kiwifruit. (A) Ethylene production pattern during ethylene-dependent ripening. Control: non-treated; Propylene: continuously treated with propylene (5000 μLL^{-1}, 22 °C) for up to 9 d; 1-MCP+Propylene: a single exposure to 1-methylcyclopropene (1-MCP, 2 μLL^{-1}) for 12 h immediately after harvest, followed by continuous propylene treatment for up to 9 d. (B) Ethylene production during low temperature-modulated ripening. Kiwifruit immediately after harvest were separately stored at either 5 °C or 22 °C in ethylene-free air for up to 49 d. Relative transcript levels of ethylene biosynthetic genes, *1-aminocyclopropane-1-carboxylate(ACC) synthase 1* (*AcACS1*, Achn364251) and *ACC oxidase 2* (*AcACO2*, Achn326461) were determined against at-harvest (0 d) samples by RT-qPCR using kiwifruit *actin* (EF063572) as an endogenous control (B, C, E, F). Data are mean (±SE) of at least three independent biological replications. Error bars not shown are smaller than the symbol used. Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

As expected, endogenous climacteric ethylene production was detected in propylene-treated fruit at a level of 0.23 nLg^{-1} h^{-1} after 5 d, which increased to 0.63 nLg^{-1} h^{-1} after 9 d (Fig. 2.4.1A). No endogenous ethylene production was measured in fruit pre-treated with 1-MCP, nor in the control fruit, throughout the experimental period. The expression of ethylene biosynthetic genes, *AcACS1* and *AcACO2*, significantly increased in propylene-treated fruit after 5 d and 9 d by 123–176- and 143–179-fold, respectively (Fig. 2.4.1B, C). in fruit pre-treated with 1-MCP there were no measurable changes in *AcACS1* expression showed, while *AcACO2* expression was significantly reduced to < 60-fold. During storage, no endogenous ethylene production was measured in healthy intact fruit either at 5 ºC or 22 ºC throughout the experimental period (Fig. 2.4.1D). There were no transcriptional changes observed in *AcACS1* (Fig. 2.4.1E), while *AcACO2* showed a considerable expression increase (65-fold) in fruit at 5 ºC after 49 d (Fig. 2.4.1F). These observations indicated that production of climacteric ethylene in kiwifruit requires ethylene signalling (triggered by propylene) and is largely dependent upon the transcriptional regulation of *AcACS1*.

2.4.3.2. Kiwifruit softening is inducible by either ethylene or low temperature

Following propylene treatment, kiwifruit firmness rapidly decreased from 73 N to 10 N within 2 d, and to < 3 N after 5 d (Fig. 2.4.2A). Fruit pre-treated with 1-MCP showed only a slight decrease in firmness, to 50 N after 9 d. The cell wall modification-associated genes, *AcPG* and *AcEXP1*, showed a marked increase in expression (> 1000- and 71-fold, respectively) during propylene treatment after 5 d and 9 d (Fig. 2.4.2B, C). The induction of both genes by propylene was significantly reduced by 1-MCP pre-treatment. Control fruit showed no significant changes in firmness, and the expression of both *AcPG* and *AcEXP1* was maintained at minimal levels throughout the experimental period.

The firmness of fruit stored at 5 ºC showed a substantial decrease from 73 N to 24 N after 21 d, and to 3 N after 49 d (Fig. 2.4.2D). At 22 ºC, fruit firmness showed only a slight decrease to 45 N after 21 d with no further changes thereafter. Both *AcPG* and *AcEXP1* expression showed sustained increases in fruit at 5 ºC, whereas their expression in fruit at 22 ºC were maintained at low levels throughout the storage period (Fig. 2.4.2E, F).

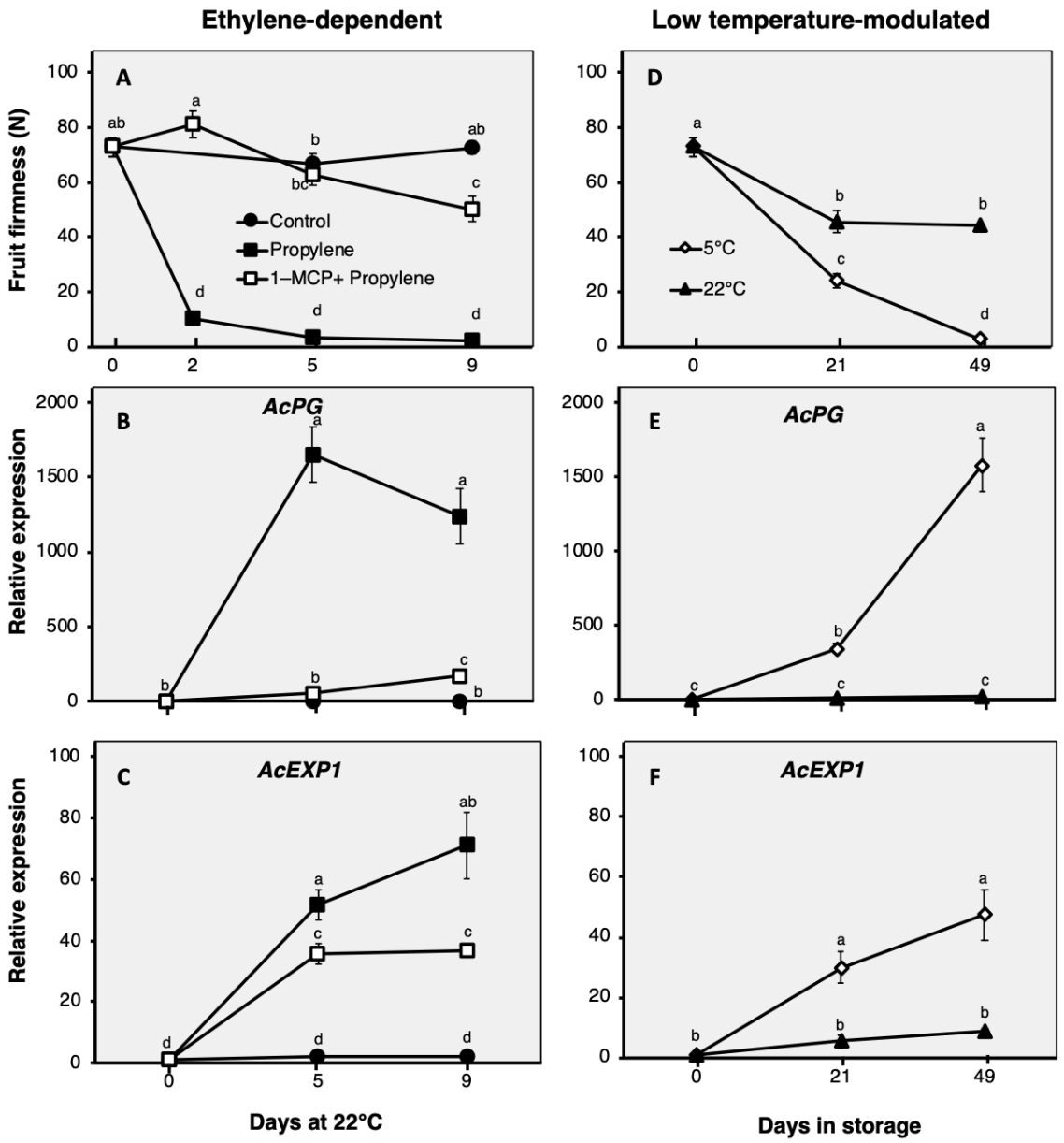


Fig. 2.4.2. Fruit softening and expression patterns of genes encoding cell wall modifying enzymes in kiwifruit. (A) Ethylene-dependent softening in kiwifruit. Control: non-treated; Propylene: continuously treated with propylene (5000 μLL^{-1}, 22 °C) for up to 9 d; 1-MCP+Propylene: a single exposure to 1-methylcyclopropene (1-MCP, 2 μLL^{-1}) for 12 h immediately after harvest, followed by continuous propylene treatment for up to 9 d. (B) Low temperature-modulated softening in kiwifruit. Kiwifruit immediately after harvest were separately stored at either 5 °C or 22 °C in ethylene-free air for up to 49 d. Relative transcript levels of kiwifruit *polygalacturonase* (*AcPG*, Achn051381) and *expansin 1* (*AcEXP1*, Achn336951) were determined against at-harvest (0 d) samples by RT-qPCR using kiwifruit *actin* (EF063572) as an endogenous control (B, C, E, F). Data are mean (±SE) of at least three independent biological replications. Error bars not shown are smaller than the symbol used. Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

2.4.3.3. Changes in the composition of major soluble sugars

The SSC of propylene-treated fruit increased rapidly from 7.8 % to a maximum 16.5 % after 5 d (Fig. 2.4.3A). This increase was significantly delayed by 1-MCP pre-treatment, with fruit showing little change for the first 5 d and a substantial increase to 13.2 % was observed after 9 d. There was a rapid increase in sucrose content of propylene-treated fruit, from 0.7 mg gFW^{-1} to 33.1 mg gFW^{-1} after 5 d, followed by a sudden decrease to 8.9 mg gFW-1 after 9 d (Fig. 2.4.3B). Both glucose and fructose contents showed a sustained increase in propylene-treated fruit to 40 mg gFW^{-1} and 36 mg gFW^{-1}, respectively, after 9 d (Fig. 2.4.3C, D). The increase in sucrose, glucose, and fructose contents by propylene treatment was significantly delayed by 1-MCP pre-treatment, although the fruit eventually attained almost similar contents at the end of the experimental period. No measurable changes in SSC, as well as the contents of sucrose, glucose, and fructose, were observed in non-treated fruit. During storage, SSC and the contents of glucose and fructose exhibited a sustained increase with no significant differences between fruit at 5 ºC and 22 ºC (Fig. 2.4.3E, G, H), while the sucrose content rose sharply in fruit at 5 ºC to a maximum 20.4 mg gFW^{-1} after 21 d followed by a decrease to 7.2 mg gFW^{-1} after 49 d (Fig. 2.4.3F). The sucrose content steadily increased in fruit at 22 ºC to 33.5 mg gFW^{-1} after 49 d.

Since the content of soluble sugars was significantly affected by propylene and storage, we examined the expression patterns of genes associated with starch degradation (*Acβ-AMY1* and *Acβ-AMY2*) and sucrose metabolism (*AcINV3-1*) (Fig. 2.4.4). *Acβ-AMY1* and *Acβ-AMY2* both showed an increase in expression in propylene-treated fruit as the soluble sugars increased, while they were significantly suppressed in fruit that were pre-treated with 1-MCP (Fig. 2.4.4A, B). During storage, there was a small increase in *Acβ-AMY1* expression in fruit at 5 ºC after 49 d, whereas no significant expression changes were observed in fruit at 22 ºC (Fig. 2.4.4D). On the other hand, *Acβ-AMY2* expression increased in fruit at both 5 ºC and 22 ºC, to a maximum (five to sevenfold) after 21 d, and later decreased after 49 d (Fig. 2.4.4E). *AcINV3-1* showed only a small expression increase (two-fold) in propylene-treated fruit after 5 d, while no significant changes were observed in fruit pre-treated with 1-MCP (Fig. 2.4.4C). However, *AcINV3-1* expression significantly increased (by five-fold after 21 d, and four-fold after 49 d) during storage of fruit at 5 ºC; no measurable changes in expression were recorded in fruit at 22 ºC (Fig. 2.4.4F).

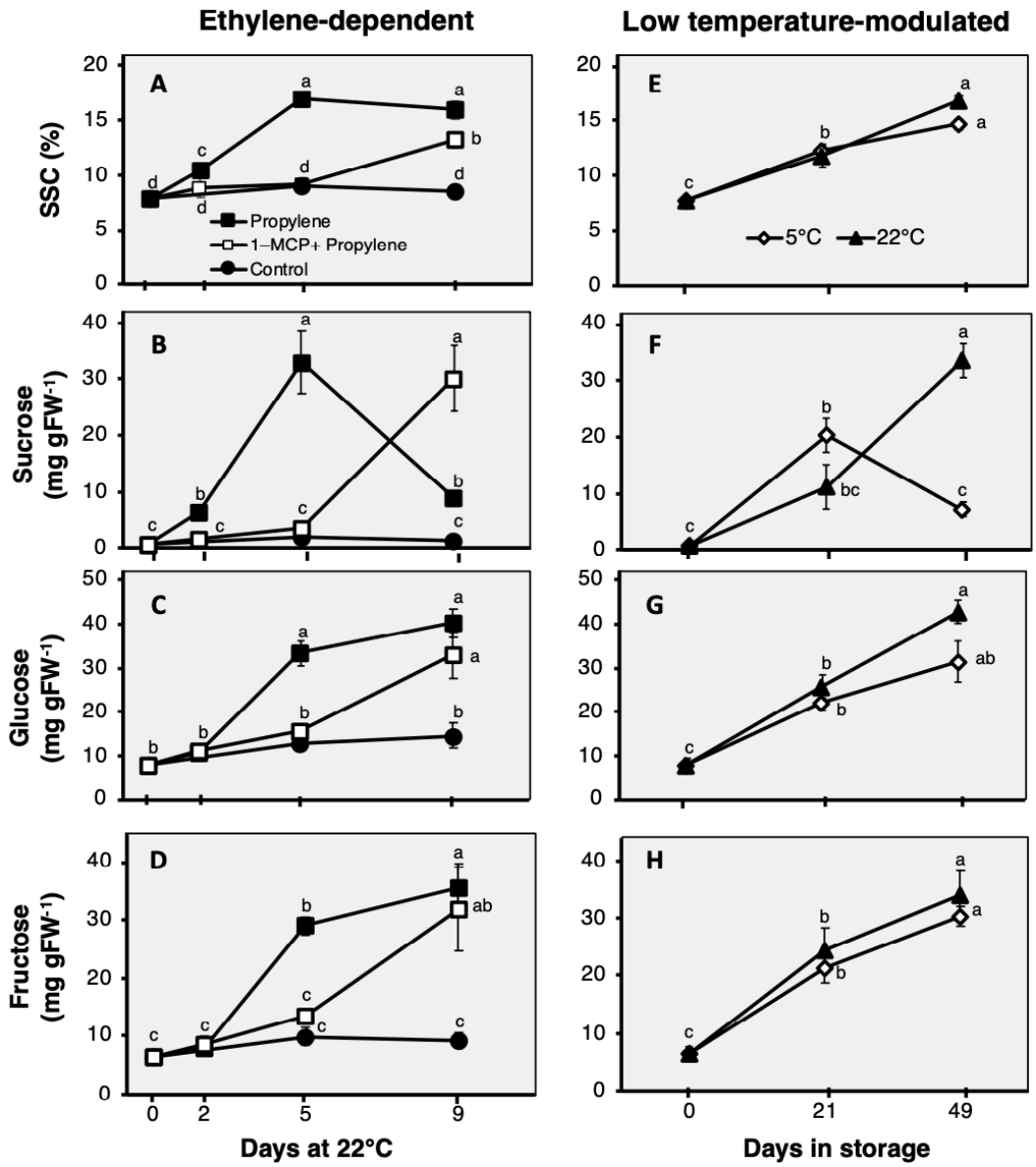


Fig. 2.4.3. Total soluble solids concentration (SSC) and composition of major sugars in kiwifruit. (A–D) Changes in SSC and concentration of sucrose, glucose and fructose during ethylene-dependent ripening. Control: non-treated; Propylene: continuously treated with propylene (5000 μLL^{-1}, 22 °C) for up to 9 d; 1-MCP+Propylene: a single exposure to 1-methylcyclopropene (1-MCP, 2 μLL^{-1}) for 12 h immediately after harvest, followed by continuous propylene treatment for up to 9 d. (E–H) Changes in SSC and concentration of sucrose, glucose and fructose during low temperature-modulated ripening. Kiwifruit immediately after harvest were stored at either 5 °C or 22 °C. Data are mean (±SE) of five independent biological replications. Error bars not shown are smaller than the symbol used. Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

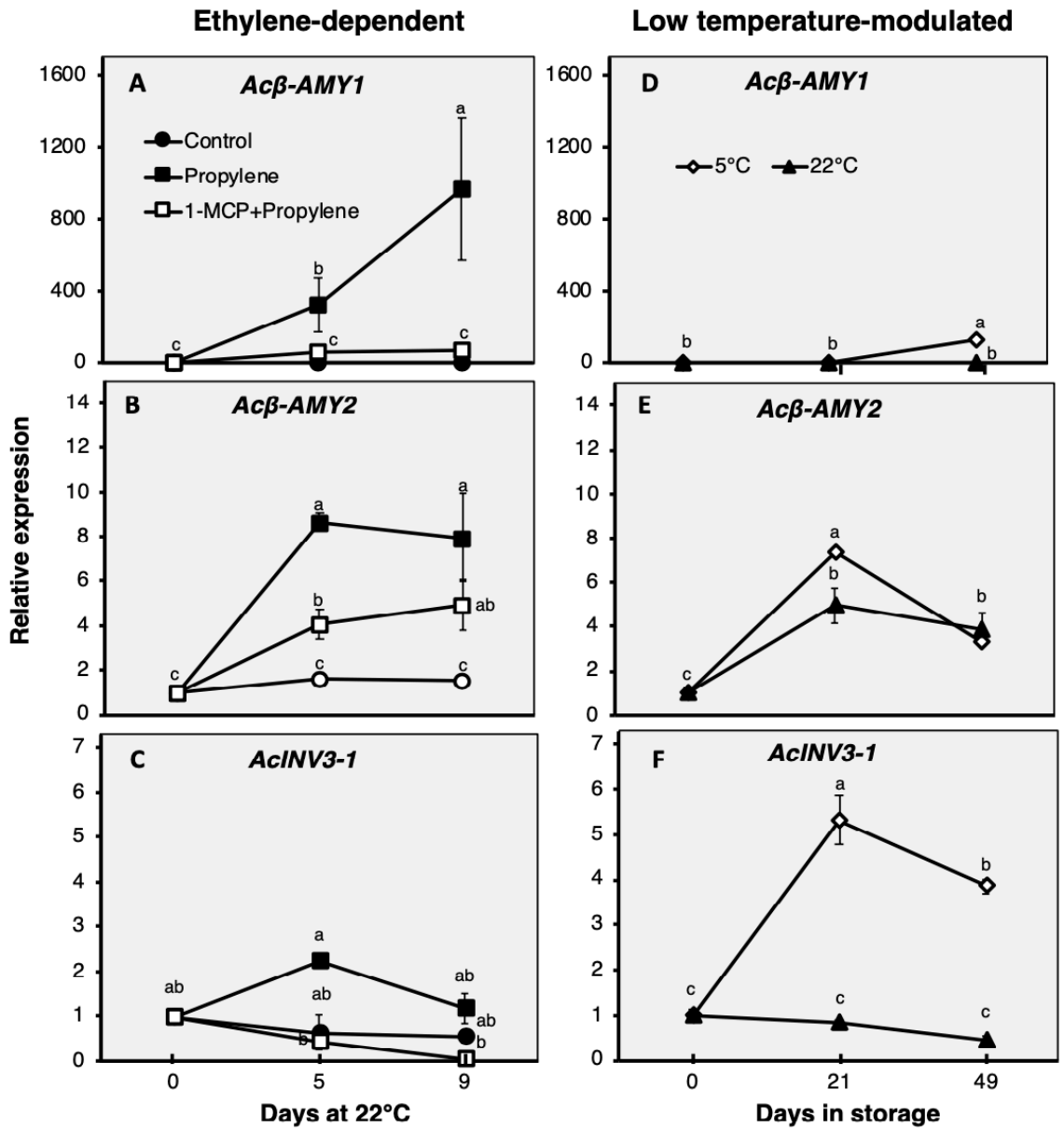


Fig. 2.4.4. Expression patterns of genes encoding starch degradation and sucrose metabolism enzymes in kiwifruit. Relative transcript levels of *β-Amylase 1* (*Acβ-AMY1*, Achn141771 and *Acβ-AMY2*, Achn212571) (A, B, D, E) and *invertase 3-1* (*AcINV3-1*, Achn319711) (C, F) were determined against at-harvest (0 d) samples by RT-qPCR using kiwifruit *actin* (EF063572) as an endogenous control. Data are mean (±SE) of three independent biological replications. Error bars not shown are smaller than the symbol used. Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

2.4.3.4. Aroma volatile production is strongly ethylene-dependent, and is undetectable during low temperature-modulated fruit ripening

Fruit aroma volatiles were identified and quantified by GC-MS during ethylene-dependent ripening, after 49 d storage at either 5 ºC or 22 ºC, and during 14 d shelf life (at 22 ºC). The major volatiles detected were esters; particularly ethyl butanoate and methyl butanoate (Fig. 2.4.5 and 2.4.61), which are considered to form part of the characteristic ripe fruit flavour for kiwifruit (Zhang *et al*., 2009; Atkinson *et al*., 2011). Propylene-treated fruit produced large amounts of ethyl butanoate at a rate of 4.2 ngg^{-1} h^{-1} after 5 d, and 20.9 ngg^{-1} h^{-1} after 9 d (Fig. 2.4.7A). Similarly, methyl butanoate was detected at a rate of 0.2 ngg^{-1} h^{-1} in propylene-treated fruit after 5 d; no further increase was observed thereafter (Fig. 2.4.7B). No measurable increase in the production of both esters was observed in fruit pre-treated with 1-MCP, as well as in control fruit. During storage, ethyl butanoate and methyl butanoate levels were undetectable in fruit at either 5 ºC or 22 ºC, while they only increased during post-storage treatment with propylene (Fig. 2.4.7C, D), consistent with endogenous ethylene production (Fig. 2.4.8A).

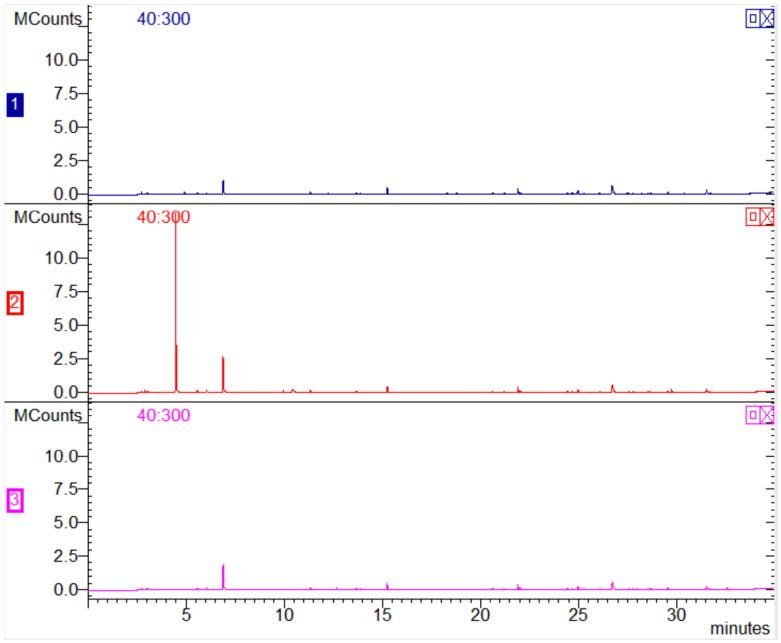


Fig. 2.4.5. A chromatogram showing the major aroma volatile compounds detected in 'Kosui' kiwifruit treated with propylene and/or 1-MCP for 9 d. Respective compounds were identified by comparing their fragmentation patterns with those from the NIST 2011 Mass Spectral Library and Software (National Institute of Standards and Technology, USA). Authentic standards were used to confirm the tentative identifications.

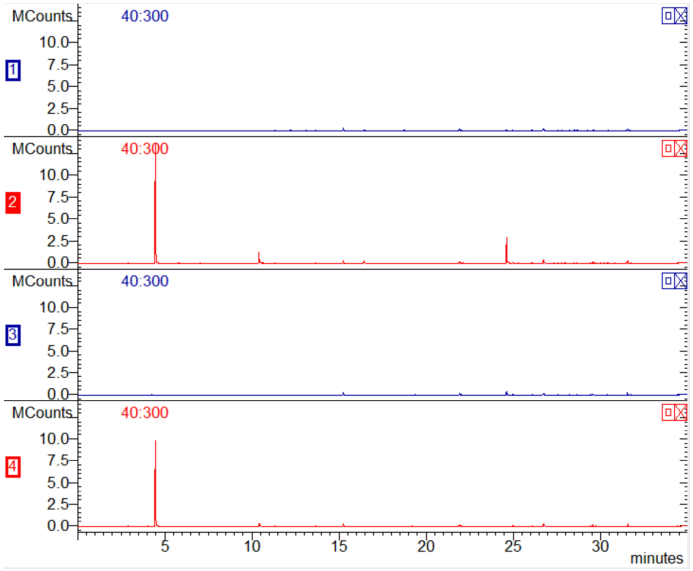


Fig. 2.4.6. A chromatogram showing the major aroma volatile compounds in 'Kosui' kiwifruit after 14 d at 22 ℃ (with or without propylene exposure) following storage at either 5 ℃ or 22 ℃ for 49 d. Respective compounds were identified by comparing their fragmentation patterns with those from the NIST 2011 Mass Spectral Library and Software (National Institute of Standards and Technology, USA). Authentic standards were used to confirm tentative identifications.

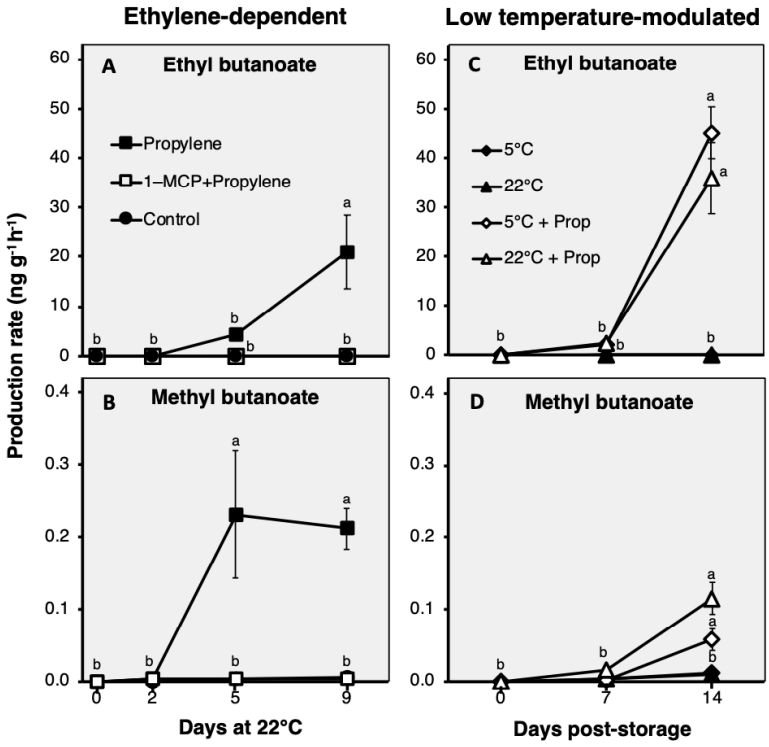


Fig. 2.4.7. Aroma volatile production patterns in kiwifruit. (A, B) Changes in production rates of ethyl and methyl butanoate during ethylene-dependent ripening. Control: non-treated; Propylene: continuously treated with propylene (5000 μLL^{-1}, 22 ℃) for up to 9 d; 1-MCP+Propylene: a single exposure to 1-methylcyclopropene (1-MCP, 2 μLL^{-1}) for 12 h immediately after harvest, followed by continuous propylene treatment for up to 9 d. (C, D) Ethyl and methyl butanoate production rates in kiwifruit after storage at either 5 ℃ or 22 ℃. After 49 d storage, fruit were transferred to 22 ℃ with or without propylene-treatment. Data are mean (±SE) of three independent biological replications containing at least three fruit each. Error bars not shown are smaller than the symbol used. Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

We also examined the expression of *AcAAT*, which encodes an alcohol acyl transferase associated with ester production during fruit ripening (Souleyre *et al*., 2005; Günther *et al*., 2011). *AcAAT* showed a dramatic increase in expression in propylene-treated fruit after 5 d (~2000-fold) and 9 d (~11,000-fold), while no significant changes were observed in fruit pre-treated with 1-MCP (Fig. 2.4.9A). By contrast, *AcAAT* showed no significant changes in expression during storage at either 5 or 22 °C, whereas it was massively up-regulated (> 10,000-fold) 7 d after post-storage treatment with propylene (Fig. 2.4.9B).

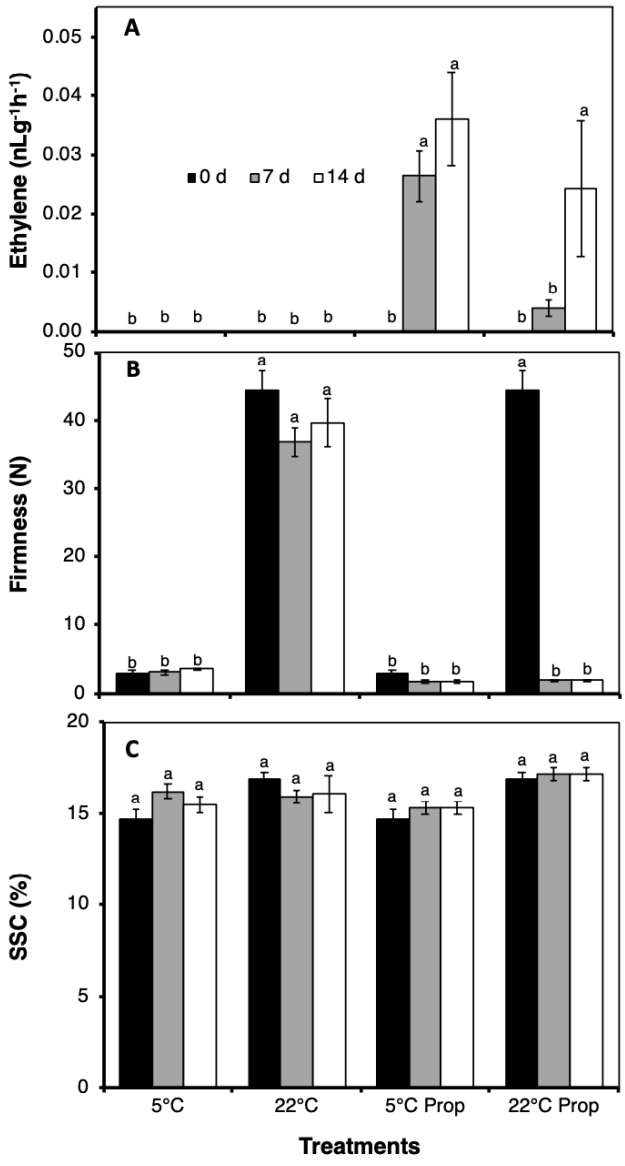


Fig. 2.4.8. Changes in ethylene production rate, firmness and SSC of 'Kosui' kiwifruit used for aroma volatile analysis. After 49 d storage period, all fruit were held at 22 °C with or without propylene treatment**.** Black, gray and white bars represent 0, 7, and 14 days post-storage. Data are mean (±SE) of five independent biological replications. Error bars not shown are smaller than the symbol used. Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

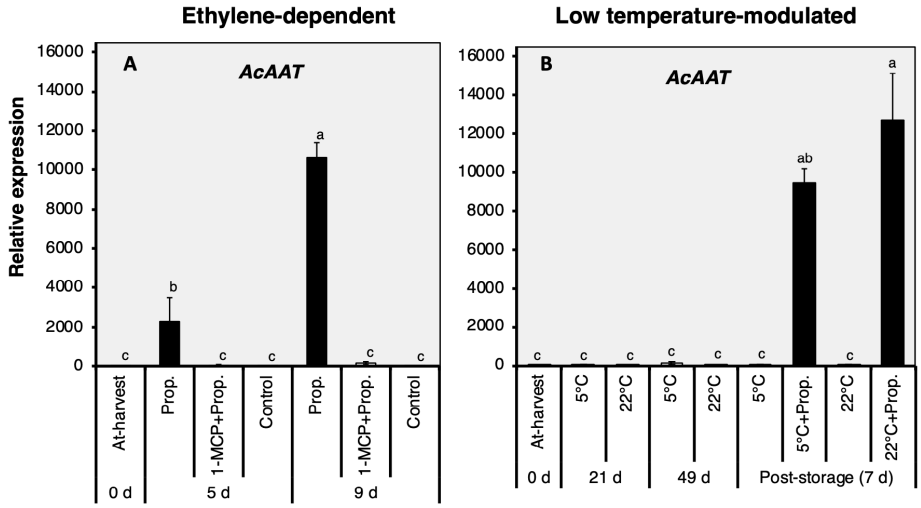


Fig. 2.4.9. Expression pattern of an alcohol acyl transferase-encoding gene in kiwifruit during ethylene-dependent and low temperature-modulated ripening. Relative transcript levels of *AcAAT* (KJ626345) were determined against at-harvest (0 d) samples by RT-qPCR using kiwifruit *actin* (EF063572) as an endogenous control. Data are mean (±SE) of three independent biological replications. Error bars not shown are smaller than the symbol used. Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

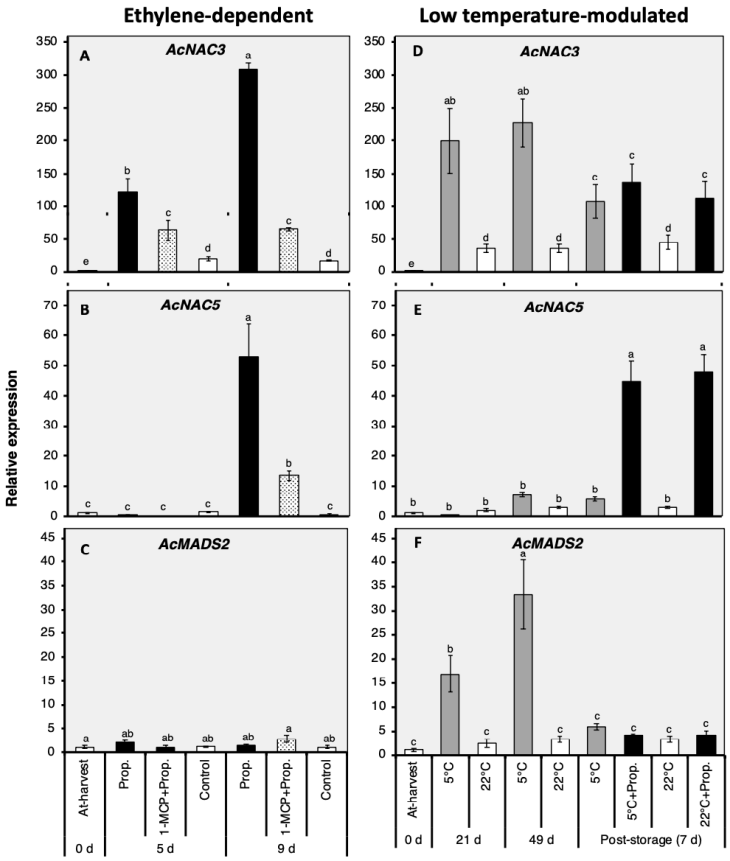


Fig. 2.4.10. Expression patterns of genes encoding ripening-associated transcription factors in kiwifruit during ethylene-dependent and low temperature-modulated ripening. Relative transcript levels of *AcNAC3* (Achn134171) (A, D), *AcNAC5* (Achn169421) (B, E) and *AcMADS2* (Achn235371) (C, F) were determined against at-harvest (0 d) samples by RT-qPCR using kiwifruit *actin* (EF063572) as an endogenous control. Data are mean (±SE) of three independent biological replications. Error bars not shown are smaller than the symbol used. Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

2.4.3.5. Expression analysis of fruit ripening-associated transcription factors

As various TFs including MADS-box and NAC domains have been shown to be key regulators of fruit ripening in kiwifruit and other fruit species (Fujisawa *et al*., 2013; McAtee *et al*., 2015; Nieuwenhuizen *et al*., 2015), we determined the expression of three TF-encoding genes (Fig. 2.4.10). *AcNAC3* was considerably up-regulated (121-fold and 309-fold after 5 d and 9 d, respectively) in propylene-treated fruit (Fig. 2.4.10A). Its expression was significantly suppressed (64-fold) in fruit pre-treated with 1-MCP, while only a small increase (18-fold) was observed in the control fruit. During storage, *AcNAC3* was also remarkably up-regulated (> 200-fold) in fruit at 5 ºC both after 21 d and 49 d, as well as after 7 d post-storage propylene treatment; only a small expression increase (< 40-fold) was observed in fruit at 22°C (Fig. 2.4.10D). A second NAC-related gene, *AcNAC5*, showed an expression increase (53-fold) 9 d after propylene-treatment, while it was significantly inhibited (14-fold) by 1-MCP pre-treatment (Figure 7B). During storage, *AcNAC5* showed no significant change in expression in fruit at either 5 ºC or 22 ºC, except in post-storage propylene-treated fruit where it was up-regulated (45-fold) after 7 d (Fig. 2.4.10E). *AcMADS2* showed a completely different expression pattern, as it showed no specific response to propylene treatment (Fig. 2.4.10C), while it was up-regulated in fruit at 5 ºC after 21 d (17-fold) and 49 d (33-fold) (Fig. 2.4.10F). There was no significant change in expression of *AcMADS2* in fruit stored at 22 ºC. It is also worth noting that *AcMADS2* expression in fruit at 5 ºC substantially dropped after transfer of the fruit to 22 ºC.

2.4.4. Discussion

The ripening behaviour of kiwifruit has elicited great interest, as substantial fruit softening during cold storage essentially occurs in the absence of any detectable ethylene; exogenous or endogenous (Antunes, 2007; Yin *et al*., 2009). In the present work, our results using 'Kosui' kiwifruit are consistent with previous research; fruit softening and the expression of cell wall modification-associated genes were induced during storage at 5 ºC (Fig. 2.4.2D–F), despite the lack of any measurable increase in ethylene production (Fig. 2.4.1D–F). It has been suggested that fruit softening during low temperature storage is brought about by basal levels of system I ethylene (Kim *et al*., 1999), based on the assumption that kiwifruit are sensitive to extremely low ethylene concentrations (Pranamornkith *et al*., 2012; Jabbar and East, 2016). However, the

present work strongly suggests that ethylene signalling is non-functional during low temperature-modulated ripening in kiwifruit.

In climacteric fruit, the ethylene biosynthetic pathway is well known to be subject to positive feedback regulation (Kende, 1993). In tomato (Barry *et al*., 2000), pear (Hiwasa *et al*., 2003), and banana (Inaba *et al*., 2007), exposure of fruit to exogenous ethylene/propylene triggers a sharp increase in ethylene production with increased expression of ethylene biosynthetic genes. In kiwifruit, the up-regulation of ethylene biosynthetic genes, *AcACS1* and *AcACO2*, by propylene exposure, coupled with their significant inhibition by 1-MCP (Fig. 2.4.1B, C), strongly indicate that ethylene biosynthesis is under the influence of ethylene signalling. These observations agree with previous reports suggesting that ethylene biosynthesis during kiwifruit ripening is regulated by a positive feedback mechanism (Whittaker *et al*., 1997; Mworia *et al*., 2010). The expression pattern of *AcACS1* suggests that it is strongly ethylene-dependent, as 1-MCP pre-treatment absolutely inhibited its induction by propylene (Fig. 2.4.1B). Of great importance is the fact that there was no measurable increase in *AcACS1* expression throughout storage of fruit at either 5 ºC or 22 ºC (Fig. 2.4.1E), which would account for the undetectable ethylene production (Fig. 2.4.1D). Therefore, lack of ethylene-dependent *AcACS1* expression, together with undetectable ethylene production during kiwifruit storage, strongly advocate for the idea that ethylene signalling is non-existent. The increase in *AcACO2* expression after 49 d at 5 ºC (Fig. 2.4.1F) is insignificant, since it has been shown using tomato that ACS regulates the rate-limiting step in ethylene biosynthesis (Yip *et al*., 1992; Wang *et al*., 2002).

It has been shown in previous studies that during fruit ripening, the production of aroma volatiles (especially esters) is regulated by the ethylene signalling pathway. Aroma volatile production was strongly inhibited in ethylene-suppressed kiwifruit (Atkinson *et al*., 2011), melon (Pech *et al*., 2008), and apple (Defilippi *et al*., 2004; Schaffer *et al*., 2007) lines. Additionally, transgenic lines treated with exogenous ethylene produced increasing concentrations of aroma volatile compounds (Schaffer *et al*., 2007; Atkinson *et al*., 2011). Another study by Defilippi *et al*. (2005) further demonstrated that the expression of *MdAAT*, together with the activity of the associated enzyme, is a rate-limiting step in ester biosynthesis in apple fruit, and both are regulated by ethylene. In the present work, propylene treatment induced the expression of *AcAAT* (Fig. 2.4.9), together with the production of ethyl butanoate and methyl butanoate in kiwifruit (Fig. 2.4.5, 2.4.7), and their complete suppression by 1-MCP confirmed that they are strongly regulated by the ethylene pathway. It is interesting that the

production of these aroma volatiles, as well as the induction of *AcAAT* expression, were not observed in fruit during storage at either 5 ºC or 22 ºC, further arguing for the lack of ethylene signalling during low temperature-modulated fruit ripening.

Fruit SSC and the concentrations of sucrose, glucose, and fructose increased in response to propylene, as well as during storage at both 5 ºC and 22 ºC (Fig. 2.4.3). These changes coincided with increased expression of *Acβ-AMY1* and *Acβ-AMY2* (Fig. 2.4.4A, B, D, E), which have been previously linked to starch degradation and sugar accumulation in kiwifruit (Nardozza *et al*., 2013; McAtee *et al*., 2015; Hu *et al*., 2016). The above observations suggest that changes in soluble sugars might involve regulatory mechanisms that are independent of both ethylene and low temperature. This is consistent with previous research using different kiwifruit cultivars (Arpaia *et al*., 1987; Boquete *et al*., 2004; Mworia *et al*., 2012; Asiche *et al*., 2018). However, *Acβ-AMY1* appears to have a stronger response to ethylene since its expression during storage is relatively low (Fig. 2.4.4A, D). Previous studies in tomatoes (Gao *et al*., 2007), melons (Pech *et al*., 2008), and apples (Defilippi *et al*., 2004) have also demonstrated that there is an ethylene-independent component in starch metabolism and sugar accumulation during fruit ripening. Nevertheless, the expression pattern of *AcINV3-1* suggests that it is more aligned to low temperature response than to ethylene, since its expression increased markedly in fruit at 5 ºC, but only slightly in response to propylene (Fig. 2.4.4C, F).

The distinction between ethylene-induced and low temperature-modulated ripening has been difficult to accomplish due to the existence of genes that respond to both stimuli, such as *AcPG* and *AcEXP1* (Fig. 2.4.2B, C, E, F). The expression of *AcNAC3* was also up-regulated by both propylene and low temperature (Fig. 2.4.10A, D), suggesting its potential role in the regulation of both ethylene-induced and low temperature-modulated ripening. However, its induction during low temperature storage is likely to be independent of ethylene, since it was previously shown that inhibiting ethylene signalling by 1-MCP failed to suppress its upregulation at 5 ºC (Asiche *et al*., 2018). NAC-related TFs are involved in regulation of several ripening-associated genes. In tomato, SlNAC4 was shown to regulate fruit ripening and carotenoid accumulation (Zhu *et al*., 2014), while in banana, several NAC TFs are induced during fruit ripening and are known to physically interact with ethylene insensitive 3 (EIN3)-like (EIL), a major component in the ethylene signalling pathway (Shan *et al*., 2012). In the present study, the observation that *AcNAC5* was exclusively induced by propylene and not by low temperature (Fig. 2.4.10B, E) challenges the notion that ethylene signalling is functional during

low temperature-modulated fruit ripening. *AcNAC5* expression correlates well with aroma volatile production patterns, suggesting its potential role in regulation of *AcAAT* and aroma volatile biosynthesis during ethylene-dependent ripening in kiwifruit. By contrast, *AcMADS2* expression was substantially induced at 5°C whereas propylene treatment failed to affect its transcript levels (Fig. 2.4.10C, F), suggesting its potential role in regulation of fruit ripening during cold storage. Our previous studies have also demonstrated the exclusive induction of *AcMADS2* by low temperature in different kiwifruit cultivars (Mitalo *et al*., 2018a; Mitalo *et al*., 2019a), confirming its alignment to regulatory mechanisms associated with low temperature response. However, since aroma volatiles were undetectable in fruit ripened by low temperature (Fig. 2.4.7C, D), it appears that *AcMADS2* does not have the capacity to bind or activate *AcAAT* or other aroma-related genes which essentially belong to the ethylene signalling pathway.

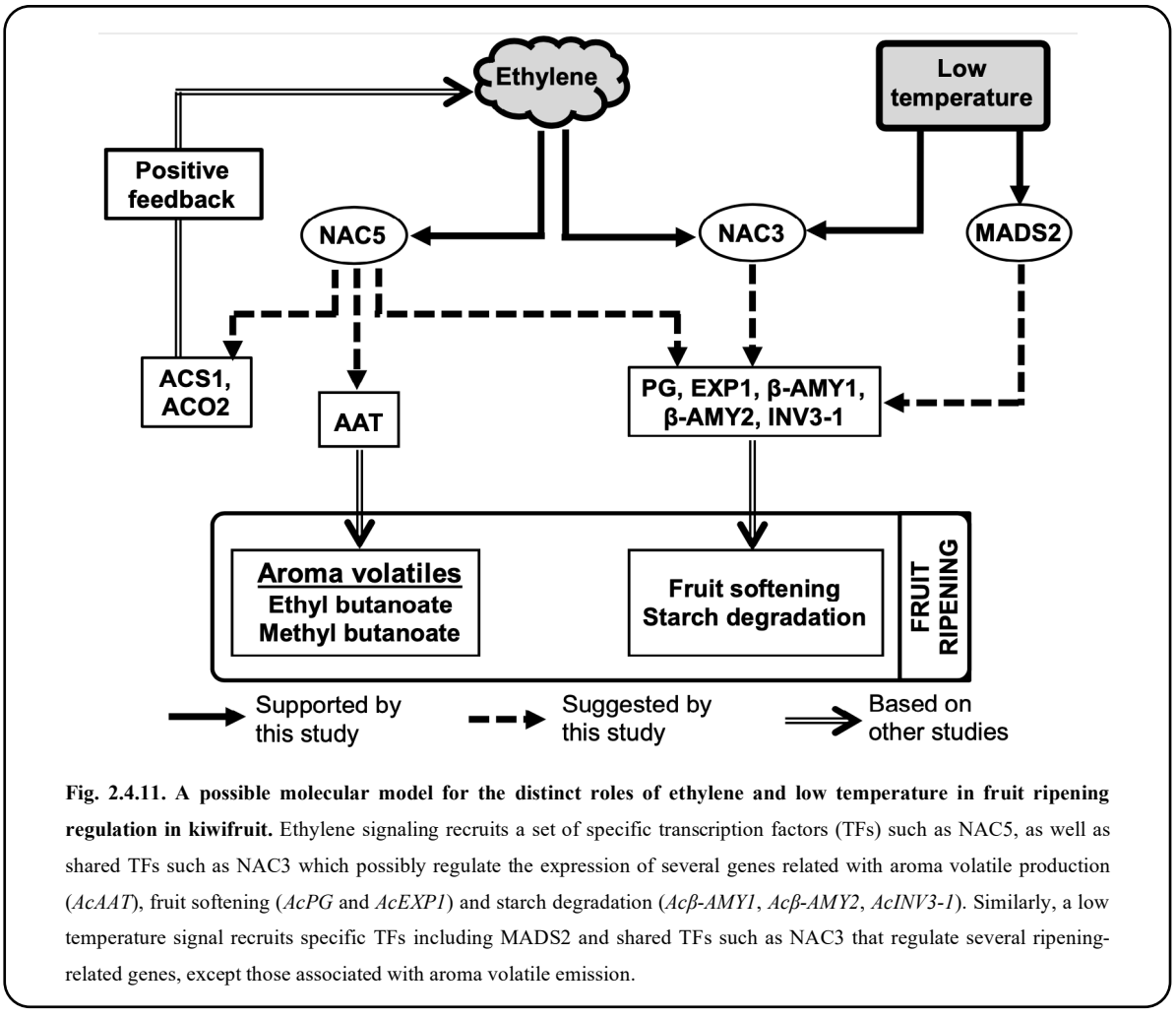


Fig. 2.4.11. A possible molecular model for the distinct roles of ethylene and low temperature in fruit ripening regulation in kiwifruit. Ethylene signaling recruits a set of specific transcription factors (TFs) such as NAC5, as well as shared TFs such as NAC3 which possibly regulate the expression of several genes related with aroma volatile production (*AcAAT*), fruit softening (*AcPG* and *AcEXP1*) and starch degradation (*Acβ-AMY1*, *Acβ-AMY2*, *AcINV3-1*). Similarly, a low temperature signal recruits specific TFs including MADS2 and shared TFs such as NAC3 that regulate several ripening-related genes, except those associated with aroma volatile emission.

2.4.5. Conclusion

In summary, the present work has demonstrated that kiwifruit ripening is inducible independently by either ethylene or low temperature signals (Fig. 2.4.11). Fruit ripened by either stimulus can attain similar quality characteristics in terms of firmness and soluble sugar levels. However, production of aroma volatiles (especially esters: ethyl butanoate and methyl butanoate) and the expression of *AcAAT* appear to be strongly dependent on the ethylene signal. These ethylene-dependent components show negligible changes during low temperature-modulated fruit ripening, providing evidence for the absence of ethylene signalling during cold storage. A distinct group of TFs such as those encoded by *AcNAC5* are exclusively induced by ethylene, suggesting their involvement in regulating ethylene-induced ripening, while a second group encoded by genes such as *AcMADS2* are exclusively aligned to low temperature response. Therefore, it appears that ethylene-induced and low temperature-modulated ripening in kiwifruit involve distinct regulatory mechanisms.

CHAPTER 3

Fruit ripening regulation in European pears by low temperature

Abstract

European pear fruit (*Pyrus communis* L.) respond to low temperature (LT) treatments by inducing ethylene production and fruit ripening. However, it is unclear to what extent this response is the result of LT alone or LT-induced ethylene production. In this study, we followed the physiological and molecular responses of 'Passe Crassane' pears to LT and the ethylene analogue, propylene, at various storage temperatures. Fruit at 20 ºC treated with propylene softened to eating firmness (13–21 N) within 9–10 d, with little changes in endogenous ethylene production (< 0.03 μg kg^{-1} s^{-1}). By contrast, LT-treated fruit (0 ºC and 5 ºC for 42 d) produced large amounts of ethylene (1–2 μg kg^{-1} s^{-1}), and rapidly softened to < 5 N after being transferred to 20 ºC. From transcriptomic analyses, we identified 437 differentially expressed genes (DEGs) between propylene-treated and control fruit, which were further augmented by LT treatment. On the other hand, the expression patterns of 763 DEGs between 5 ºC vs. 20 ºC was not significantly affected by propylene treatment in non-chilled fruit. To examine LT-induced and ethylene-induced pathways separately during chilling, the responses of LT-induced DEGs to 1-methylcyclopropene (1-MCP), an ethylene action inhibitor, were assessed. Among the 763 LT-induced DEGs, 1-MCP treatment disrupted the expression of 390 DEGs, indicating that they were regulated by LT-induced ethylene. Intriguingly, 373 DEGs including transcription factor-related genes such as *PcERF98-like*, *PcATL65*, *PcMYB6-like*, *PcGRP2-like*, *PcTCP7* and *PcMBF1c* were unaffected by 1-MCP treatment, and thus, likely to be influenced by LT alone. Based on these results, the potential role of these LT-specific genes/pathways as a key factor modulating changes in ethylene production and responsiveness leading to fruit ripening in European pears is discussed.

3.1. Introduction

Fleshy fruit ripening is achieved through multifaceted metabolic processes that are regulated by both internal and external cues. In climacteric fruit such as tomatoes, apples and pears, a vast majority of fruit ripening-associated processes are regulated by the phytohormone ethylene (Giovannoni, 2004; Klee and Giovannoni, 2011; Xu *et al*., 2012). These fruit show a marked increase in ethylene production rate (autocatalytic ethylene) during ripening, which regulates various processes such as fruit softening, starch degradation to sugars, pigment accumulation and aroma volatile production (Klee and Giovannoni, 2011; Xu *et al*., 2012; Osorio *et al*., 2013). Ethylene is biosynthesized from *S*-adenosyl-methionine (SAM) via two

successive reactions catalysed by 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) (Wang *et al*., 2002). Both ACS and ACO are encoded by members of multigene families (Wang *et al*., 2002; Cherian *et al*., 2014).

European pear fruit (*Pyrus communis* L.) require ethylene both for the induction and progression of fruit ripening (Lelièvre *et al*., 1997b; Itai *et al*., 1999; Hiwasa *et al*., 2003). However, low temperature (LT) treatment can also enhance the ripening rate in a cultivar-dependent manner (Gerasopoulos and Richardson, 1997; Villalobos-Acuña and Mitcham, 2008). Early-maturing cultivars such as 'Bartlett' and 'La France' have short (0–21 d) LT treatment requirements (Hiwasa *et al*., 2003; Nham *et al*., 2017), while late-maturing cultivars such as 'Passe Crassane' require long durations (≥ 40 d) before they are capable of ripening (Lelièvre *et al*., 1997b; El-Sharkawy *et al*., 2003; El-Sharkawy *et al*., 2004). Indeed, non-chilled 'Passe Crassane' fruit fail to ripen even after 145 d at room temperature (El-Sharkawy *et al*., 2004).

The expression of ethylene biosynthetic genes, *PcACS1*, *PcACS2* and *PcACO1*, rapidly increase during and after LT treatment, and their respective enzyme activities account for the rise in ethylene production during fruit ripening at room temperature (Lelièvre *et al*., 1997b; El-Sharkawy *et al*., 2004; Fonseca *et al*., 2005). Ethylene biosynthetic genes, together with ACS and ACO activities were also induced by chilling exposure in 'Granny Smith' and 'Braeburn' apple cultivars (Jobling *et al*., 1991; Lelièvre *et al*., 1995; Tian *et al*., 2002). In addition, the expression of cell wall modification-associated genes including *POLYGALACTURONASE 1* (*PcPG1*), *PcPG2*, *β-GALACTOSIDASE* (*Pcβ-GAL*) and *ENDO-1,4-BETA-GLUCANASE 2* (*PcEG2*) increases in LT-treated pears (Hiwasa *et al*., 2003; Fonseca *et al*., 2005). Their suppression by post-cold 1-methylcyclopropene (1-MCP) treatment indicates that they are regulated by LT-induced ethylene (Lelièvre *et al*., 1997b; Hiwasa *et al*., 2003; El-Sharkawy *et al*., 2004). These observations indicate that the primary function of LT in pear fruit ripening is to initiate ethylene production. Although *PcACS1* and *PcACS2* do not respond to ethylene/propylene treatment in non-chilled 'Passe Crassane' fruit, their induction after LT-exposure is repressed by 1-MCP treatment (Lelièvre *et al*., 1997b; El-Sharkawy *et al*., 2004). This indicates that LT exposure enables *ACS* genes to respond to ethylene signalling; however, the molecular mechanisms that lead to this change in ethylene sensitivity remain to be fully elucidated.

LT regulates fruit ripening in kiwifruit independent of ethylene signalling (Mworia *et al*., 2012; Asiche *et al*., 2018; Mitalo *et al*., 2018a; Mitalo *et al*., 2018b; Mitalo *et al*., 2019a). Fruit softening and the expression of associated genes were accelerated during storage at 5 ºC in the absence of autocatalytic ethylene production, while they were not suppressed by repeated 1-MCP treatments. Follow-up transcriptome analysis revealed that genes encoding several transcription factors (TFs) were exclusively induced by LT (Asiche *et al*., 2018). Furthermore, ethylene-independent induction of fruit softening and *MdPG1* expression by low temperature was reported in 'Royal Gala' apples (Tacken *et al*., 2010). A recent study in 'Bartlett' pears demonstrated that various TF-encoding genes were also regulated by LT during fruit ripening (Nham *et al*., 2017). However, the expression of some genes in this class including *AGAMOUS-LIKE 24* (*PcAGL24*) and *TEOSINTE BRANCHED1 CYCLOIDEA PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR1 9b* (*PcTCP9b*) was also altered in non-chilled fruit producing substantial amounts of ethylene, indicating that they were not LT-specific.

Our objective was to investigate whether LT-specific genes function in European pear fruit ripening. Ethylene responses in 'Passe Crassane' fruit before, during and after LT treatment were followed. The transcriptomic data of 'Passe Crassane' fruit treated with propylene at 20 ºC, as well as those stored in air at either 5 ºC or 20 ºC for 42 d, were analysed. Additionally, we attempted to isolate LT-specific responses by using 1-MCP treatment to block ethylene signalling. Based on these analyses, we suggest a model in which LT-specific genes play a central role in the modulation of both ethylene-biosynthetic and ethylene-regulated genes to enhance fruit ripening.

3.2. Materials and methods

3.2.1. Plant material

'Passe Crassane' pear fruit grown under standard cultural practices were harvested from a commercial orchard in Akaiwa, Japan at a physiological maturity stage (195 d after full bloom, DAFB). Fruit were harvested both in 2015 (flesh firmness: 49 N, soluble solids concentration [SSC]: 12.23 %, titratable acidity [TA]: 0.38 %) and in 2016 (flesh firmness: 72 N, SSC: 12.20 %, TA: 0.27 %). Careful sorting of fruit was carried out to exclude those with physical

injuries and disease symptoms, and to ensure uniform size before randomly assigning them to treatments.

'Bartlett' pear fruit were harvested in 2016 at a physiological maturity stage (119 DAFB; firmness: 70 N; SSC: 11 % and TA: 0.42 %) from a commercial orchard in Tsuruoka, Japan. After careful sorting, the fruit were divided into two sets of twenty corresponding to the treatments.

3.2.2. Treatments

3.2.2.1. Propylene treatment

In both 2015 and 2016, two sets of 10 fruit each were used in this experiment. The first set was placed in gas-tight plastic containers that were continuously treated with 5000 µL L^{-1} propylene for up to 9 d (2015) and 10 d (2016). Treatments with propylene, a well-known ethylene analogue (McMurchie *et al*., 1972; Mworia *et al*., 2010; Asiche *et al*., 2016), were carried out to trigger the ethylene signal, and to enable the determination of endogenous ethylene. The second set contained non-treated fruit as a control. All groups (propylene-treated and control) were held at 20 ºC. Soda lime was placed in plastic containers during propylene treatments to reduce CO_2 accumulation in the containers.

3.2.2.2. Storage tests

In 2015, fruit were stored at either 5 ºC (50 fruit) or 20 ºC (20 fruit) to compare their ripening rates (Appendix 2). After detecting substantial endogenous ethylene levels (after 42 d storage), fruit at 5 ºC were divided into four groups of ten. The first group were treated with 2 µL L^{-1} 1-MCP for 12 h to keep them insensitive to ethylene before being transferred to 20 ºC for 6 d, while the second group was transferred to 20 ºC without any treatment. The third and fourth groups were maintained at 5 ºC for 6 d with or without 1-MCP treatment. 1-MCP was released by dissolving SmartFresh™ powder (AgroFresh, PA, USA) in water as previously described (Mworia *et al*., 2012).

In 2016, four groups of 20 fruit were stored at either 0 °C, 5 °C, 10 °C or 20 °C for 42 d (Appendix 3). An additional group of 20 fruit were also stored at 5 ºC with regular 1-MCP treatments (once a week). After 42 d storage, fruit in the respective groups were transferred to

20 ºC for 6 d to assess their ripening characteristics. Separately, 'Bartlett' pear fruit (20 fruit each) were stored at either 5 ºC or 20 ºC for up to 20 d.

3.2.3. Evaluation of fruit ripening characteristics

Ethylene production was determined as described in our previous work (Mworia *et al*., 2012). Briefly, individual fruit were incubated (at the respective storage temperatures) in a 1360-mL container for up to 1 h. Ethylene production rate was measured by withdrawing 2 mL headspace gas and injecting it into a gas chromatograph (model-GC4 CMPF, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (set at 200 ºC) and an activated alumina column (set at 80 ºC). Values were expressed as a mean of five fruit. Flesh firmness was determined at four different equatorial regions of peeled fruit using a penetrometer (model SMT-T-50, Toyo Baldwin, Tokyo, Japan) fitted with an 8-mm plunger. Data were recorded as Newtons (N) and firmness was expressed as a mean of three fruit. SSC of the fruit juice was determined using a digital Atago PR-1 refractometer (Atago Co. Ltd, Tokyo, Japan), and the values were expressed as a percentage. TA was determined by titrating the fruit juice against 0.1 N NaOH, and the values were expressed as percentage citric acid equivalents. After the above assessments, flesh tissue from three fruit was cut into small pieces and stored at -80 ºC until further analysis.

3.2.4. Phytohormone content analysis

Phytohormones, including indole-3-acetic acid (IAA), abscisic acid (ABA), jasmonic acid (JA), jasmonoyl-*L*-isoleucine (JA-Ile), gibberellins A_1 (GA_1), gibberellins A_4 (GA_4), *trans*-zeatin (tZ), N6-isopentenyladenine (iP) and salicylic acid (SA), were quantified according to the method of Gupta *et al*. (2017) with slight modifications. Briefly, frozen fruit tissue (~200 mg) were immersed in 4 mL of 80 % (v/v) acetonitrile containing 1 % (v/v) acetic acid and known amounts of stable isotope-labelled internal standards, thoroughly ground in a few minutes in a mortar and stored for 1 h at 4 °C to extract the hormones. Tissue debris was pelleted by centrifugation at 3000 ×g for 10 min, and the pellet was washed with 80 % (v/v) acetonitrile containing 1 % (v/v) acetic acid. The two supernatants were combined, evaporated in a vacuum centrifugal evaporator (Genevac miVac Centrifugal Concentrator, SP Scientific, PA, USA), and dissolved in 1 % (v/v) acetic acid. The extracted hormones were loaded onto a reverse-phase solid-phase extraction cartridge (Oasis HLB 1 cc; Waters Corporation, MA, USA). The cartridge was washed with 1 mL of 1 % acetic acid and hormones were eluted with 2 mL of 80 % acetonitrile containing 1 % acetic acid. The eluent was evaporated to leave the extracts

in 1 mL of 1 % acetic acid and subjected to cation exchange chromatography on an Oasis MCX 1-cc extraction cartridge (Waters Corporation, MA, USA). The acidic fraction was eluted with 1 mL of 80 % acetonitrile containing 1 % acetic acid. A portion of the acidic eluate was analysed for SA. The cartridge was further washed with 5 % aqueous ammonia, and the basic fraction was eluted with 40 % acetonitrile containing 5 % ammonia and analysed for tZ and iP. The remaining acidic fraction was evaporated, dissolved in 1 % acetic acid, and loaded onto an Oasis WAX 1-cc extraction cartridge (Waters Corporation, MA, USA). The cartridge was further washed with 1 % acetic acid and the remaining hormones were eluted with 80 % acetonitrile containing 1 % acetic acid. The eluate was analysed for IAA, GA_1, GA_4, ABA, JA, and JA-Ile.

All fractions were analysed on an Agilent 1260-6410 Triple Quad LC/MS system (Agilent Technologies, CA, USA) equipped with a ZORBAX Eclipse XDB-C18 column (Agilent Technologies, USA). The conditions for liquid chromatography are described in Appendix 4. The multiple-reaction-monitoring mode of the tandem quadrupole mass spectrometer and precursor-product ion transitions for each compound are listed in Appendix 5. The content of each phytohormone was expressed on a fresh weight basis.

3.2.5. RNA extraction

Total RNA was extracted from ~2 g ground tissue of each of three fruit using the hot borate method (Wan and Wilkins, 1994), with slight modifications. DNase I (Nippon Gene, Tokyo) treatment followed by clean-up using FavorPrep after Tri-Reagent RNA Clean-up Kit (Favorgen Biotech Co., Ping-Tung, Taiwan) were carried out to remove genomic DNA contamination from the total RNA.

3.2.6. RNA-seq, differential expression and functional annotation analyses

Fruit samples collected in 2015 were used for RNA-seq analyses. Non-chilled fruit samples included control (collected immediately after harvest) and propylene (continuously treated with propylene at 20 ºC for 9 d) samples. Storage samples included fruit at 5 ºC and 20 ºC (stored for 42 d), 5 °C + 1-MCP (treated with 1-MCP immediately after 42 d storage at 5 ºC) and 5 °C→20 °C (transferred from 5 ºC to 20 ºC). Illumina paired-end sequencing libraries (three independent biological replications) were constructed using NEBNext® Ultra™ RNA Library Prep Kit for Illumina (New England BioLabs, MA, USA). Library quality was checked

with Bioanalyzer Agilent High Sensitivity Chip (Agilent Technologies, CA, USA) according to the manufacturer's instructions. The libraries were sequenced using Illumina HiSeq 2500 platform (Hokkaido System Co., Ltd. Japan).

Mapping and read count procedures were conducted using CLC genomic workbench (CLC Bio-Qiagen, Aarhus, Denmark) according to the procedure by Akagi *et al*. (2014). Reads trimming was done to obtain ≥ 10 million paired reads for each sample before mapping them onto *Pyrus communis* Genome v1.0 Draft Assembly and Annotation (Chagné *et al*., 2014). Functional grouping was performed using the Pyrus Genome Database, the online BLAST function of NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and InterPro v69 (https://www.ebi.ac.uk/interpro/). Mapped reads of each gene were processed to reads per kb per million (RPKM), and then used for detection of differentially expressed genes (DEGs) based on false discovery rates (FDR) test, using edgeR (Robinson *et al*., 2010). Selection of DEGs was based on two criteria: (i) genes with RPKM values ≥ 5.0 and FDR values ≤ 0.001 in any of the treatments; (ii) fold change ≥ 3.0 in average RPKM for propylene vs. control, and/or 5 °C vs. 20 °C samples. 1-MCP effect was determined as ≥ 2-fold increase or decrease in 1-MCP-treated vs non-treated samples.

3.2.7. Validation by reverse-transcriptase quantitative PCR (RT-qPCR)

RT-qPCR analyses were carried out using fruit samples collected in 2016. First strand cDNA was synthesized from 2.4 µg total RNA using RevTraAce reverse transcriptase (Toyobo, Osaka, Japan) and random hexamer primers, according to the manufacturer's instructions. Gene-specific primers were designed using Primer3 (version 0.4.0; http://bioinfo.ut.ee/primer3-0.4.0/) for 24 genes (Appendix 6). Gene expression of three fruit was examined on a MyiQ Single-Color Reverse Transcriptase-Quantitative PCR Detection System (Bio-Rad, Hercules, CA, USA) using TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. *PcActin* (*PCP000431*) was used as the housekeeping gene after examining its constitutive expression pattern from the RNA-seq results. The specificity of all primers was verified by melting curve analysis. Relative gene expression values were calculated using the $2^{-\Delta\Delta Ct}$ method with samples at harvest (0 d) calibrated as 1.

Additionally, the expression patterns of selected DEGs from the RNA-seq results were further examined in 'Bartlett' pear fruit stored at either 5 ºC or 20 ºC for 12 d and 20 d (coinciding with the detection of endogenous ethylene production) using RT-qPCR.

3.2.8. Statistical analysis

Statistical analyses were carried out using R version 3.4.0 software package (R Project). ANOVA followed by post-hoc Tukey's tests ($p \leq 0.05$) were used to detect differences in fruit ripening characteristics, gene expression and phytohormone content among the various treatments.

3.3. Results

3.3.1. Effect of propylene treatment on fruit ripening in non-chilled pears

In both years, non-chilled 'Passe Crassane' fruit showed little change of ethylene production during propylene treatment, with only 0.01–0.03 μg kg^{-1} s^{-1} after 9–10 d (Fig. 3.1A). However, propylene-treated fruit showed a massive decrease in flesh firmness from the initial 49 N to 13 N after 9 d in 2015, and from 72 N to 21 N after 10 d in 2016 (Fig. 3.1B). Propylene-treated fruit did not exhibit any noticeable changes in either SSC or TA (Appendix 7). These findings indicate that while non-chilled 'Passe Crassane' fruit can respond to ethylene/propylene by softening rapidly, they are incapable of producing autocatalytic ethylene.

3.3.2. Effect of storage temperature on fruit ripening

Ethylene production was detected in fruit stored at 5 ºC at a level of 0.2–0.4 μg kg^{-1} s^{-1} after 42 d, and it rapidly increased to over 1.7 μg kg^{-1} s^{-1} 5 d after being transferred to 20 ºC (Fig. 3.2A). In 2015, a single 1-MCP exposure before transferring fruit from 5 ºC to 20 ºC substantially reduced ethylene production from 1.7 μg kg^{-1} s^{-1} to 0.4 μg kg^{-1} s^{-1}. In 2016, repeated 1-MCP treatments completely suppressed ethylene production during storage at 5 ºC. Fruit stored at 0 ºC in 2016 also produced detectable ethylene levels (~0.3 μg kg^{-1} s^{-1}) after 42 d and upon being transferred to 20 ºC. No measurable ethylene production was detected in fruit stored at either 10 ºC or 20 ºC.

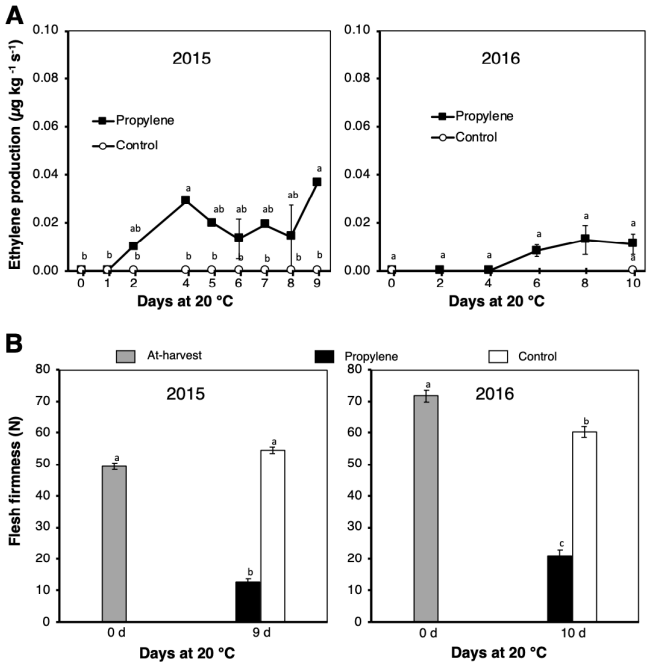


Fig. 3.1. Ethylene production rates (A) and flesh firmness (B) of non-chilled 'Passe Crassane' pear fruit treated with propylene during 2015 and 2016 seasons. Propylene treatment (5000 µL L^{-1}) was carried out continuously at 20 ºC. Values are means (± SE) of five individual biological replications (five fruit) for ethylene production rate and three individual biological replications (three fruit) for flesh firmness. Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

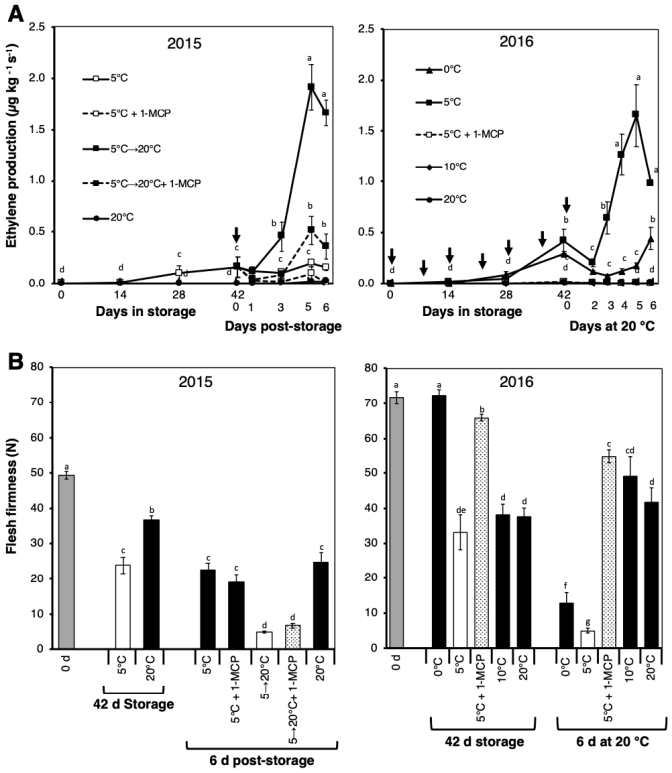


Fig. 3.2. Ethylene production rates (A) and flesh firmness (B) of 'Passe Crassane' pear fruit after 42 d storage at various temperatures, and 6 d post-storage. Arrows in (**A**) indicate points at which 1-MCP treatments (2 µL L^{-1} for 12 h) were carried out. Values are means (± SE) of five individual biological replications (five fruit) for ethylene production rate and three individual biological replications (three fruit) for flesh firmness. Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

Concomitant with ethylene production patterns, the flesh firmness of fruit stored at 5 ºC decreased gradually to 24–33 N after 42 d storage, and then rapidly to 5 N after 6 d of being transferred to 20 ºC (Fig. 3.2B). The decrease in flesh firmness of fruit at 5 ºC was inhibited by either single or repeated 1-MCP treatments. In 2016, the flesh firmness of fruit stored at 0 ºC did not change significantly after 42 d, but decreased to 13 N after transferring the fruit to 20 ºC. Fruit at both 10 ºC and 20 ºC also showed slight softening patterns during storage although their flesh firmness was higher than that of fruit at 0 ºC and 5 ºC. No particular storage temperature-dependent changes in fruit SSC and TA were observed throughout the experimental period (Appendix 7). Collectively, these findings indicate that LT treatments (0 ºC and 5 ºC for 42 d) can sufficiently trigger fruit ripening (defined as softening) in pears by enabling the fruit to produce autocatalytic ethylene. Since 1-MCP treatment effectively inhibited LT-induced softening, we used 1-MCP-treated fruit at 5 ºC in subsequent experiments to isolate molecular changes that may act in ethylene-induced or LT-induced pathways.

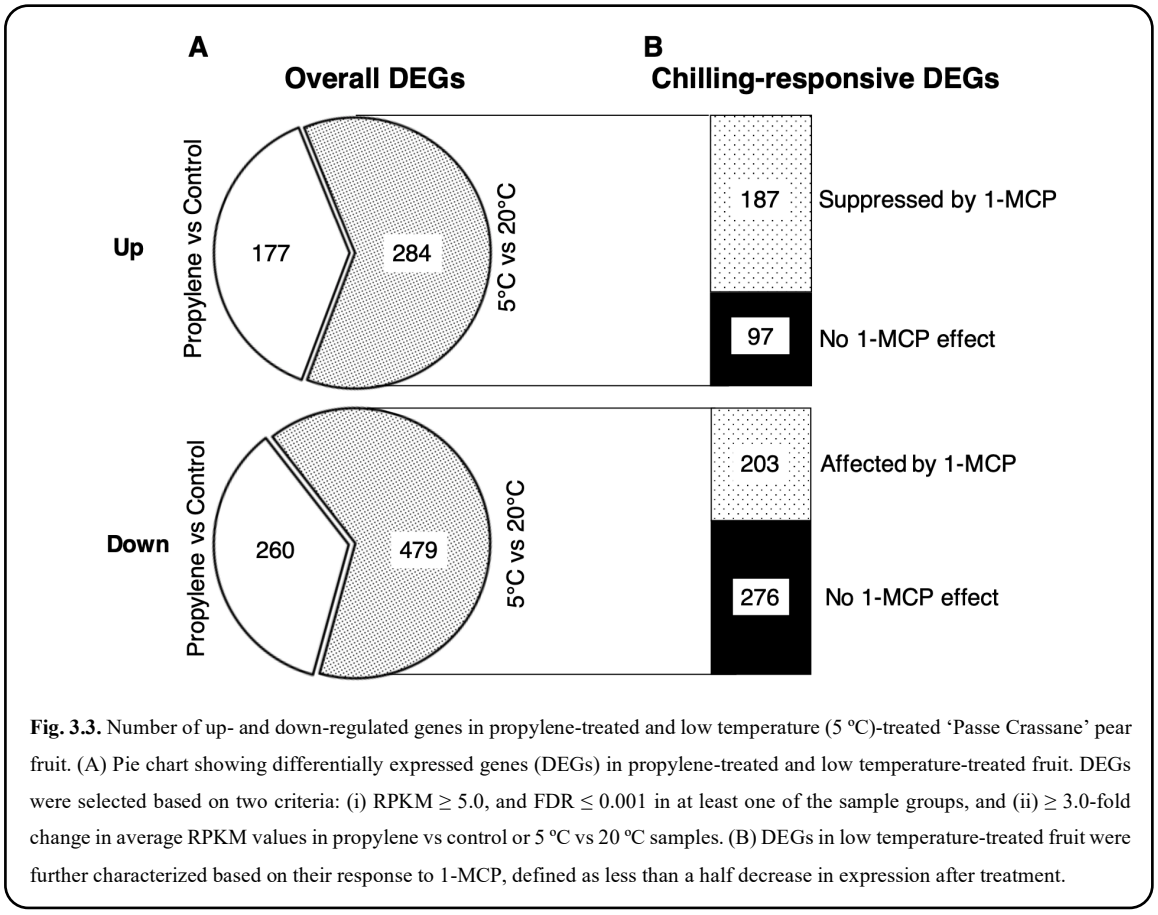


Fig. 3.3. Number of up- and down-regulated genes in propylene-treated and low temperature (5 ºC)-treated 'Passe Crassane' pear fruit. (A) Pie chart showing differentially expressed genes (DEGs) in propylene-treated and low temperature-treated fruit. DEGs were selected based on two criteria: (i) RPKM ≥ 5.0, and FDR ≤ 0.001 in at least one of the sample groups, and (ii) ≥ 3.0-fold change in average RPKM values in propylene vs control or 5 ºC vs 20 ºC samples. (B) DEGs in low temperature-treated fruit were further characterized based on their response to 1-MCP, defined as less than a half decrease in expression after treatment.

3.3.3. Overview of RNA-seq analysis

RNA-seq was used to examine transcriptional changes triggered by propylene treatment in non-chilled fruit, as well as those triggered by LT treatment (Fig. 3.3). In total, 1,200 DEGs were detected in response to either propylene or LT treatments (q-value < 0.001, Fig. 3.3A). Out of these, propylene treatment up-regulated 177 DEGs and down-regulated 260 DEGs (q-value < 0.001, Fig. 3.3A), of which most showed more drastic responses to LT treatment. The other DEGs (N=763) did not respond to propylene treatment at all, while they were either up-regulated (N=284) or down-regulated (N=479) between 5 ºC and 20 ºC (Fig. 3.3B). Of the 284 LT-up-regulated DEGs, 187 genes were significantly repressed by 1-MCP treatment. Similarly, of the 479 LT-down-regulated DEGs, 203 genes had their expression level restored by 1-MCP treatment. Intriguingly, 97 LT-up-regulated DEGs and 276 LT-down-regulated DEGs were not significantly altered by 1-MCP treatment.

Altogether, the DEGs obtained in this study were placed into three categories. The first category constituted of genes that were regulated by propylene treatment in non-chilled fruit and represent molecular ethylene responses that may or may not require LT treatment. The second category comprised genes that did not respond to propylene treatment in non-chilled fruit but were regulated by LT treatment and 1-MCP altered their expression patterns. This category represents molecular ethylene responses that emerged after LT exposure. Finally, the third category consisted of genes that did not respond to propylene treatment in non-chilled fruit but were regulated by LT treatment with 1-MCP having no effect on their expression. These genes represent LT-induced molecular changes that fall outside the scope of ethylene influence.

3.3.4. Identification and expression analysis of genes associated with ethylene biosynthesis

As ethylene production of non-chilled propylene-treated fruit and low temperature-stored fruit markedly differed, we sought to identify all the genes encoding key biosynthetic enzymes ACS and ACO in the European pear genome. Nineteen *ACS-like* genes were identified (Fig. 3.4A), of which only four (*ACS12*: *PCP013369*, *ACS10*: *PCP022032*, *ACS2*: *PCP013122* and *ACS1*: *PCP011500*) were expressed (Fig. 3.4B). Among the four, *PcACS12* showed a constitutive expression pattern, *PcACS10* was down-regulated by all treatments, while *PcACS2* was slightly up-regulated by propylene, and during storage at 20 ºC (Fig. 3.4B). By contrast, *PcACS1* was exclusively up-regulated at 5 ºC, and the induction at 5 ºC was substantially suppressed by a

single 1-MCP treatment. Of the twenty-nine *ACO-like* genes identified (Fig. 3.5A), only four were differentially expressed, all of which showed a similar pattern, that is, up-regulated by either propylene or low temperature with higher expression values at low temperature (Fig. 3.5B).

The expression patterns of *PcACS1* and *PcACO1*(*PCP011683*) were further verified by RT-qPCR analysis using the 2016 fruit samples (Fig. 3.6). *PcACS1* showed insignificant expression changes in non-chilled propylene-treated fruit, while its transcripts massively accumulated in fruit at 0 ºC and 5 ºC (Fig. 3.6A). Furthermore, the accumulation of *PcACS1* transcripts in fruit at 5 ºC was prevented by regular 1-MCP treatments. *PcACO1* expression was induced by propylene treatment in non-chilled fruit (236-fold), with significantly higher transcript levels in fruit at 0 ºC (700-fold) and 5 ºC (2000-fold) (Fig. 3.6B). It should be noted that the up-regulation of *PcACO1* in fruit at 5 ºC was also abolished by regular 1-MCP treatments.

3.3.5. Expression analysis and functions of genes regulated by ethylene in non-chilled fruit

A major category of DEGs identified by RNA-seq included transcripts that were regulated by propylene treatment in non-chilled fruit (Fig. 3.3A). Since propylene is a well-known ethylene analogue (McMurchie *et al*., 1972; Mworia *et al*., 2010), genes in this category were considered to be regulated by ethylene without any low temperature requirements. Main functional groups in this category included Metabolism (26.1 %), Protein Biosynthesis and Modification (12.4 %), Regulation of Transcription (8.7 %), Transport (5.5 %), Stress (4.1 %), Redox Homeostasis (2.7 %), Hormones (2.5 %), Signal Transduction (0.6 %), Electron Transport (0.6 %) and Others (20.4 %). To verify the RNA-seq results, we selected six specific genes including those associated with cell wall modifications (*PcPG1*, *Pcβ-GAL2*, *EXPANSIN 9*: *PcEXP9* and *PECTATE LYASE 18a*: *PcPL18a*), starch degradation (*α-AMYLASE*: *Pcα-AMY*) and TFs (*PcGRAS2*) for further analysis via RT-qPCR (Fig. 3.7). These analyses confirmed that *PcPG1*, *PcPL18a*, *Pcβ-GAL2*, *PcEXP9* and *Pcα-AMY* were all significantly up-regulated (Fig. 3.7A–E), while *PcGRAS2* was significantly down-regulated (Fig. 3.7F) in propylene-treated fruit. The expression patterns of these genes were also altered in fruit stored at both 0 ºC and 5 ºC while no significant changes were observed at either 10 ºC or 20 ºC. Notably, transcript levels of *PcPL18a*, *Pcβ-GAL2*, *PcEXP9* and *Pcα-AMY* in fruit at 0 ºC and 5 ºC were considerably higher than in non-chilled propylene-treated fruit (Fig. 3.7B–E). It should also be noted that

repeated 1-MCP treatments altered the expression patterns of genes in this category during storage at 5 ºC.

3.3.6. Transcripts responding to ethylene after LT exposure

The bulk of DEGs identified by RNA-seq (63.6 %) did not respond to propylene treatment in non-chilled fruit but were either up-regulated (284) or down-regulated (479) by LT (Fig. 3.3A). As LT-stored fruit produced substantial ethylene, we speculated that ethylene-regulated genes were key members of this group. To test this hypothesis, we examined the effect of blocking ethylene signalling by 1-MCP treatment on their expression pattern. Indeed, 1-MCP treatment altered the expression of 187 up-regulated genes and 203 down-regulated genes (Fig. 3.3B), indicating that they are under ethylene regulation. Dominant functional groups in this category were Metabolism (26.4 %), Protein Biosynthesis and Modification (13.8 %), Regulation of Transcription (10.8 %), Transport (9 %), Stress (6.4 %), Hormones (3.1 %), Redox Homeostasis (2.8 %), Signal Transduction (1.8 %), Photosynthesis (1 %), Electron Transport (0.8 %) and Others (21.5 %). Eight representative genes in this category including those associated with oxidative reactions (*2-OXOGLUTARATE-DEPENDENT DIOXYGENASE*: *Pc2OGD*), ethylene perception (*ETHYLENE RESPONSE 2*: *PcETR2*), cell wall modifications (*PcEG3*, *PcEXPA1-like*, *PcPL18b*), auxin signalling (*SMALL AUXIN UP RNA21-like*: *PcSAUR21-like*) and TFs (*PcMADS2* and *BRASSINAZOLE RESISTANT 1*: *PcBZR1*) were verified by RT-qPCR analysis. Expectedly, these genes showed no significant changes in expression levels in non-chilled propylene-treated fruit (Fig. 3.8). Nonetheless, there was a marked accumulation of *Pc2OGD*, *PcETR2*, *PcEG3*, *PcEXPA1-like*, *PcPL18b* and *PcSAUR21-like* transcripts during storage at 0 ºC and 5 ºC, while 1-MCP treatment repressed their expression at 5 ºC (Fig. 3.8A–F). Storage of fruit at 0 ºC and 5 ºC noticeably down-regulated *PcMADS2* and *PcBZR1*, while 1-MCP treatment rescued their expression (Fig. 3.8G, H). Collectively, these findings indicate that although this gene category are initially unresponsive to ethylene in non-chilled fruit, LT treatments enabled ethylene to regulate their expression.

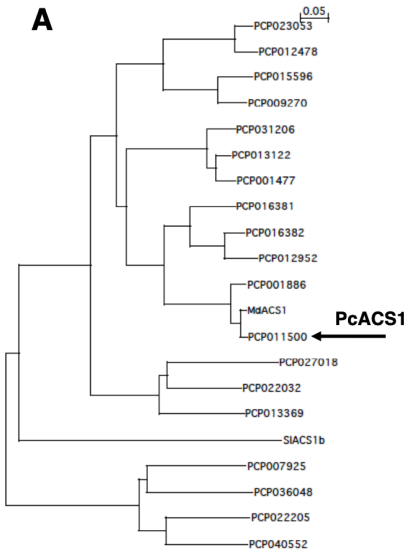
3.3.7. Identification of LT-specific genes and expression analysis of selected genes

The remaining part of LT-induced DEGs (97 up-regulated and 276 down-regulated) did not show any response to 1-MCP treatment (Fig. 3.3B), indicating that they were independent of ethylene. The most dominant functional groups in this class were Metabolism (20.9 %), Protein

Biosynthesis and Modification (16.3 %), Regulation of Transcription (14.7 %) and Transport (8.3 %). Other minor functional groups included Stress (4.3 %), Photosynthesis (2.9 %), Redox Homeostasis (2.9 %), Hormones (2.7 %), Signal Transduction (2.7 %), Electron Transport (1.6 %) and Calcium Binding (1.3 %). The expression of eight representative genes, including those associated with auxin transport (*WALLS ARE THIN 1-LIKE*: *PcWAT1-like*), chlorophyll metabolism (*PHOTOSYSTEM II PROTEIN 1*: *PcPSBO1*), TFs (*ETHYLENE RESPONSE FACTOR 98-LIKE*: *PcERF98-like*, *RING-H2 FINGER ATL65*: *PcATL65*, *PcMYB6-like*, *GLYCINE-RICH PROTEIN 2-LIKE*: *PcGRP2-like*, *PcTCP7*, and *MULTIPROTEIN BRIDGING FACTOR 1c*: *PcMBF1c*), were further examined using RT-qPCR (Fig. 3.9). None of the genes in this category exhibited a significant change in their expression patterns in non-chilled propylene-treated fruit. However, transcripts of *PcWAT1-like*, *PcERF98-like*, *PcATL65* and *PcMYB6-like* exclusively accumulated during storage at 0 ºC and 5 ºC, while 1-MCP treatment failed to repress their up-regulation at 5 ºC (Fig. 3.9A–D). However, *PcGRP2-like*, *PcTCP7*, *PcPSBO1* and *PcMBF1c* expression was exclusively down-regulated during storage at 0 ºC and 5 ºC, with 1-MCP treatment also failing to abolish their down-regulation at 5 ºC (Fig. 3.9E–H). Together, these findings indicate that genes in this category neither respond to ethylene before nor after chilling, but they are regulated by LT alone.

3.3.8. Expression analysis of LT-specific genes in 'Bartlett' pear fruit

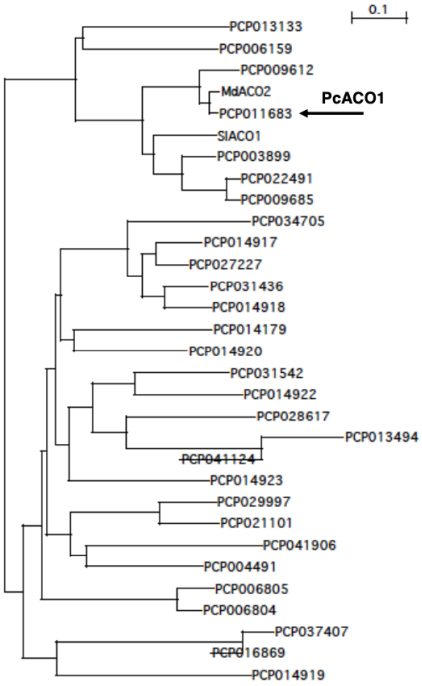
To further test the possibility that LT-specific genes identified in 'Passe Crassane' fruit are independent of ethylene, we examined the expression patterns of selected genes in 'Bartlett' pear fruit during storage at either 5 °C or 20 °C (Fig. 3.10). 'Bartlett' fruit at 5 °C or 20 °C exhibited almost similar ethylene production rates, ~1.4 µg kg^{-1} s^{-1} after 12 d and > 10 µg kg^{-1} s^{-1} after 20 d (Fig. 3.10A). However, fruit at 5 ºC showed high expression levels of *PcWAT1-like* and *PcERF98-like* after 12 d and 20 d, whereas no significant expression changes were registered at 20 ºC at the same time-points (Fig. 3.10B, D). Similarly, *PcGRP2-like* and *PcPSBO1* expression was significantly down-regulated in fruit at 5 ºC, while there were no significant changes at 20 ºC (Fig. 3.10C, E). These findings clearly confirm that the expression of *PcWAT1-like*, *PcERF98-like*, *PcGRP2-like* and *PcPSBO1*, as well as other genes in the same category, is undeniably regulated by LT independent of ethylene.



B

Gene ID	0 d	Prop.	5 °C	5 °C+1-MCP	20 °C
PCP013369	2.80	2.74	2.02	1.60	3.36
PCP022032	4.18	1.53	1.51	1.81	1.71
PCP027018	0.78	0.64	0.97	0.82	0.76
PCP031206	0.00	0.02	0.01	0.12	0.09
PCP036048	0.00	0.00	0.00	0.00	0.00
PCP040552	0.00	0.00	0.00	0.00	0.00
PCP001477	0.00	0.00	0.00	0.00	0.02
PCP001886	0.00	0.01	0.13	0.00	0.00
PCP007925	0.00	0.00	0.00	0.00	0.00
PCP009270	0.00	0.00	0.00	0.00	0.00
PCP011500	0.47	0.78	95.47	4.10	0.54
PCP012478	0.00	0.00	0.00	0.00	0.00
PCP012952	0.00	0.01	0.00	0.00	0.00
PCP013122	0.03	1.16	0.02	0.52	2.77
PCP015596	0.07	0.00	0.00	0.02	0.00
PCP016381	0.00	0.00	0.00	0.00	0.00
PCP016382	0.00	0.01	0.00	0.03	0.01
PCP022205	0.00	0.00	0.00	0.00	0.00
PCP023053	0.00	0.00	0.02	0.02	0.00

Fig. 3.4. Identification and expression analysis of 'Passe Crassane' pear *ACS-like* genes. **A.** Phylogenetic alignment of predicted proteins of *ACS-like* genes identified in the European pear genome alongside those of SlACS1 (Solyc08g081550) and MdACS1 (DQ137849). **B.** Expression analysis of RNA-seq reads of *ACS-like* genes at harvest (0 d), after propylene treatment at 20 ºC for 9 d (Prop.), and storage at either 5 ºC or 20 ºC for 42 d.



B

Gene ID	0 d	Prop.	5 °C	5°C+1-MCP	20 °C
PCP003899	5.21	3.74	10.42	15.65	4.73
PCP004491	0.04	4.06	0.48	1.97	3.35
PCP006159	0.02	0.03	0.11	0.02	0.10
PCP006804	0.58	0.34	0.06	0.09	0.13
PCP006805	41.60	53.52	64.74	42.05	43.20
PCP009612	38.09	5245.28	9418.40	6225.77	1879.26
PCP009685	0.32	1.77	0.79	0.09	0.15
PCP011683	6.05	102.44	221.28	110.17	35.88
PCP013133	0.00	0.00	0.03	0.00	0.00
PCP013494	13.31	1711.61	4493.62	2276.63	659.71
PCP014179	0.01	0.01	0.00	0.00	0.00
PCP014917	0.27	0.03	0.00	0.00	0.00
PCP014918	1.76	0.98	0.00	0.00	2.64
PCP014919	0.65	0.38	0.02	0.03	1.24
PCP014920	2.83	2.99	1.16	1.62	3.53
PCP014922	0.03	0.00	0.01	0.00	0.14
PCP014923	0.62	0.06	0.10	0.32	0.08
PCP016869	12.87	11.16	9.01	11.45	13.76
PCP021101	0.00	0.00	0.00	0.00	0.01
PCP022491	0.49	0.35	0.05	0.00	0.00
PCP027227	0.20	0.00	0.00	0.00	0.18
PCP028617	0.00	0.00	0.00	0.00	0.00
PCP029997	39.93	6.99	0.99	8.73	17.13
PCP031436	0.00	0.02	0.00	0.00	0.01
PCP031542	1.96	1.74	1.13	1.05	1.75
PCP034705	0.00	0.00	0.00	0.15	0.00
PCP037407	0.13	0.00	0.00	0.00	0.00
PCP041124	0.00	0.00	0.00	0.00	0.00
PCP041906	1.90	0.67	0.10	0.05	1.34

Fig. 3.5. Identification and expression analysis of 'Passe Crassane' pear *ACO-like* genes. **A.** Phylogenetic alignment of predicted proteins of *ACO-like* genes identified in the European pear genome alongside those of SlACO1 (Solyc07g049530) and MdACO2 (AF015787). **B.** Expression analysis of RNA-seq reads of *ACO-like* genes at harvest (0 d), after propylene treatment at 20 ºC for 9 d (Prop.), and storage at either 5 ºC or 20 ºC for 42 d.

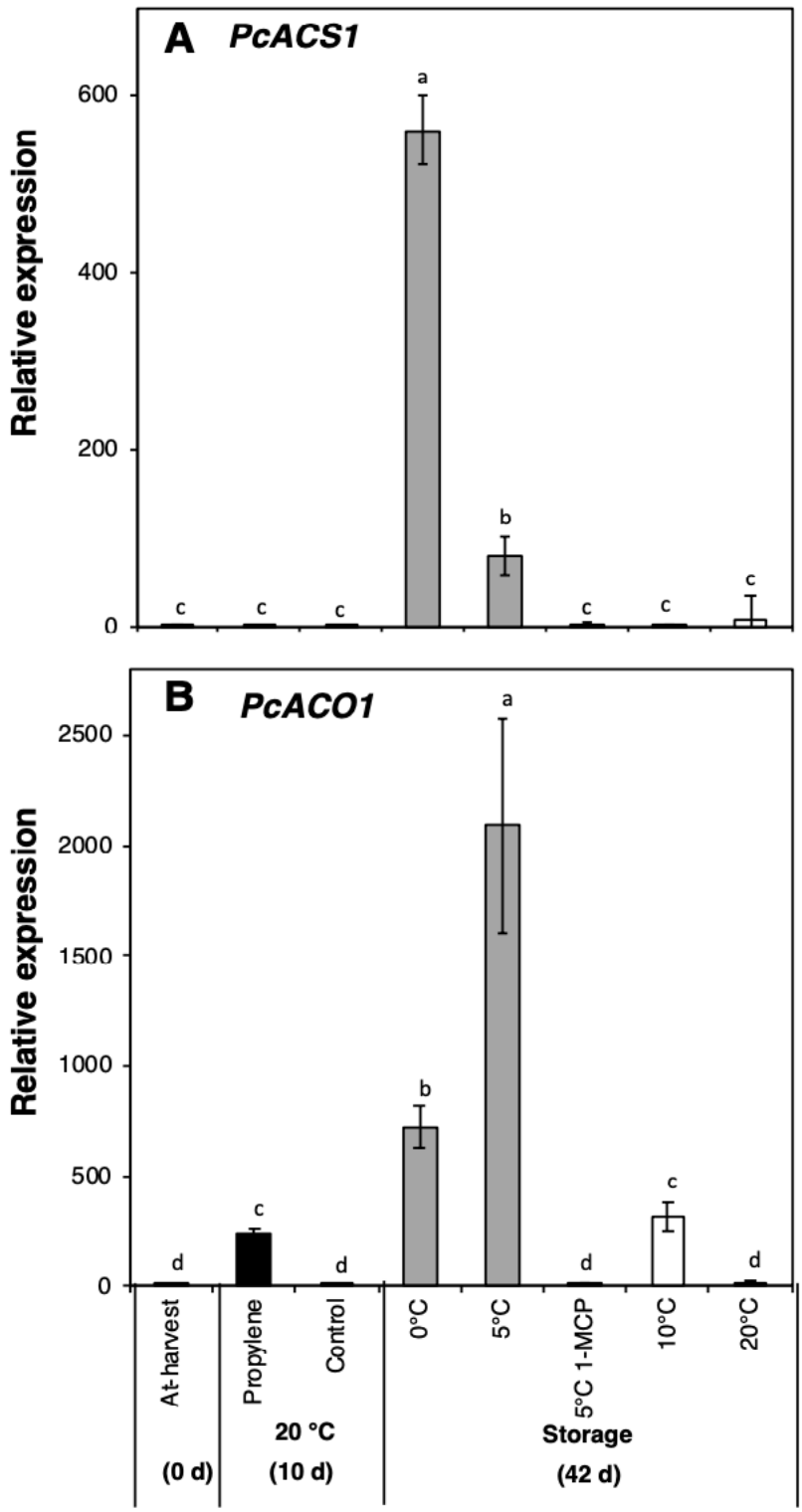


Fig. 3.6. Relative expression changes and RT-qPCR validation of selected 'Passe Crassane' pear genes associated with ethylene biosynthesis. Expression values were determined relative to *PcActin* with samples at harvest (0 d) set as 1. Values represent means (± SE) of three independent biological replications obtained from the 2016 experiment. Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

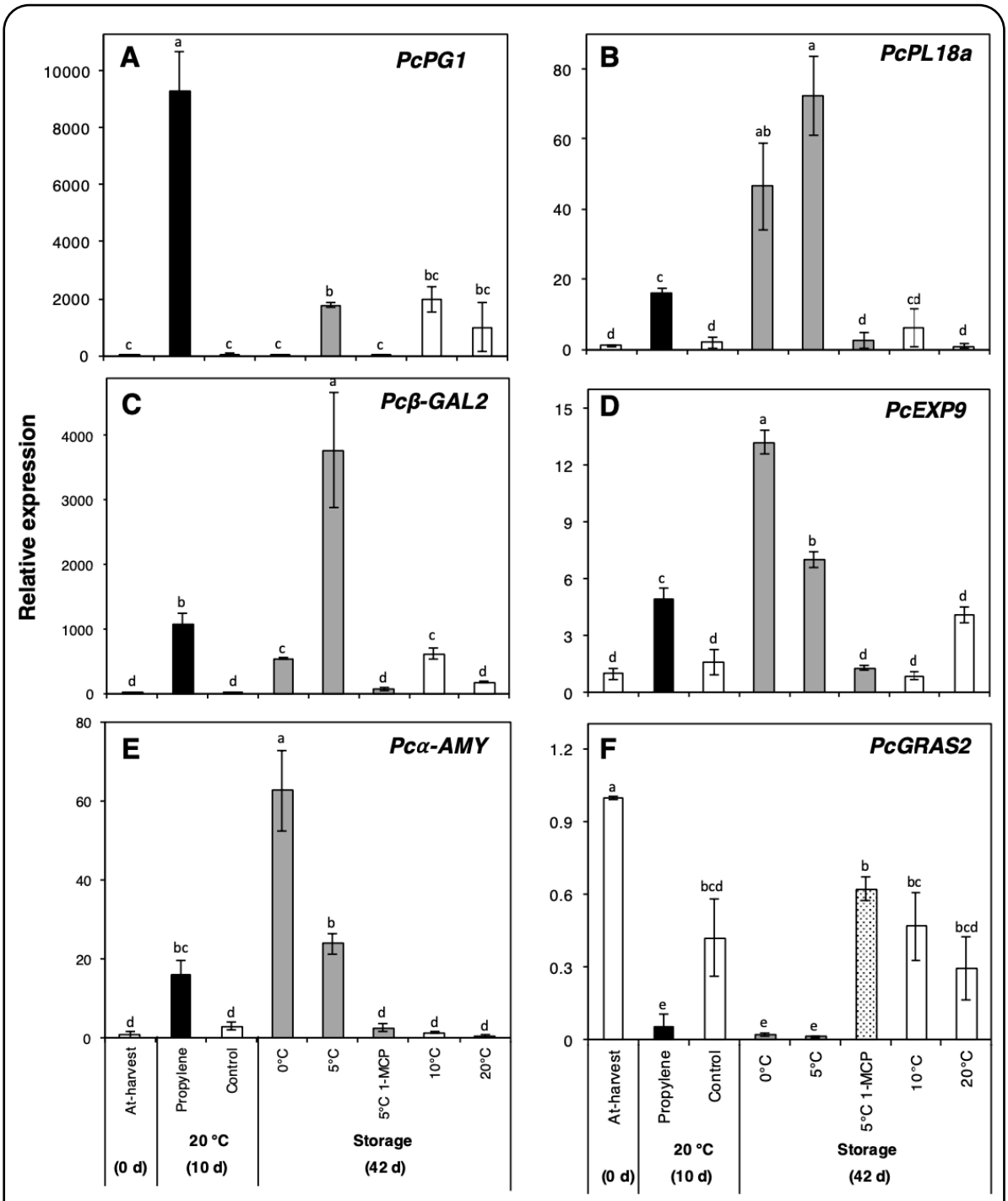


Fig. 3.7. Relative expression changes and RT-qPCR validation of selected 'Passe Crassane' pear genes responding to either propylene treatment in non-chilled fruit or low temperature storage. Expression values were determined relative to *PcActin* with samples at harvest (0 d) set as 1. Values represent means (± SE) of three independent biological replications obtained from the 2016 experiment. Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

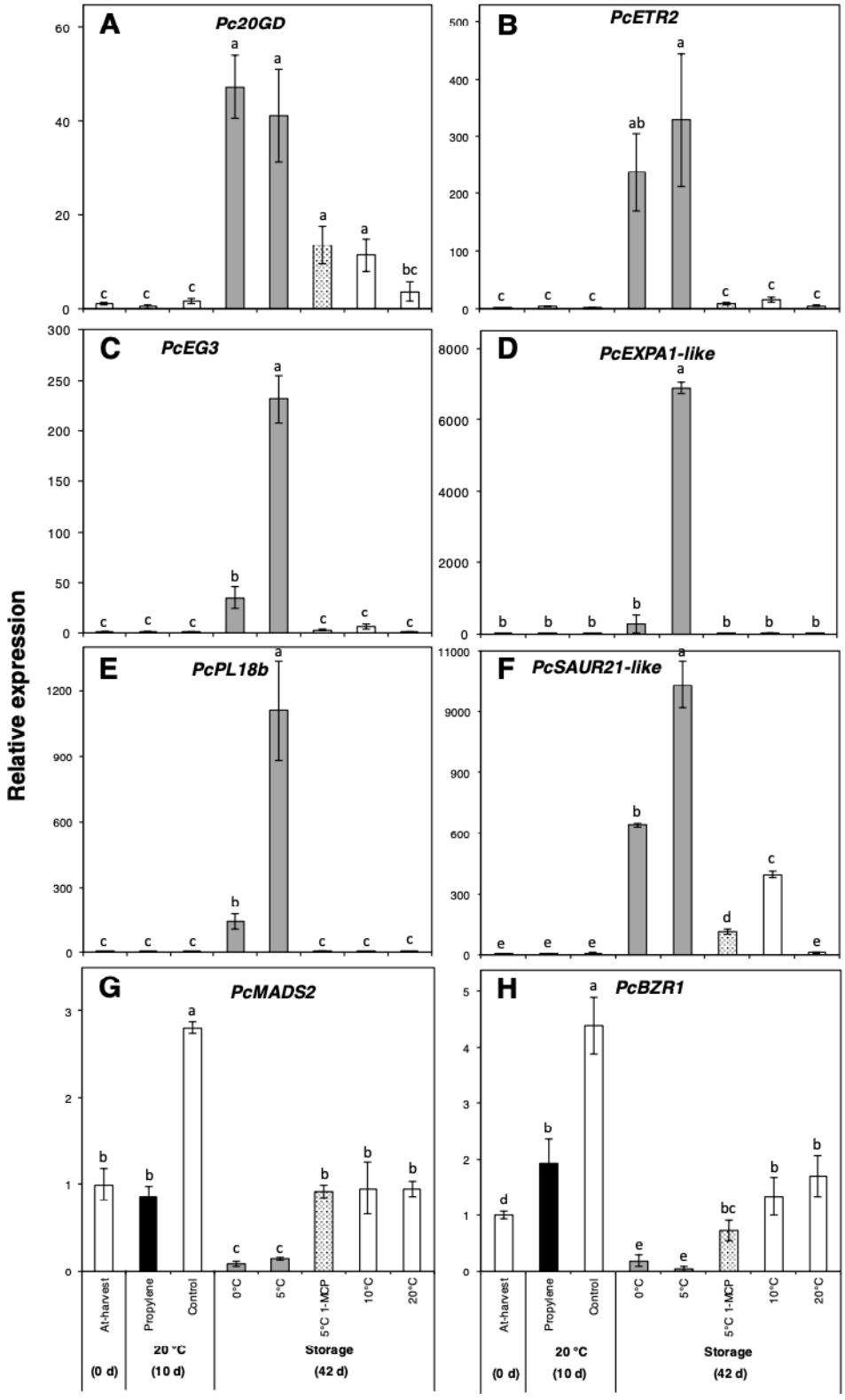


Fig. 3.8. Relative expression changes and RT-qPCR validation of selected 'Passe Crassane' pear genes responding to ethylene (i.e. altered by 1-MCP treatment) exclusively after low temperature exposure. Expression values were determined relative to *PcActin* with samples at harvest (0 d) set as 1. Values represent means (± SE) of three independent biological replications obtained from the 2016 experiment. Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

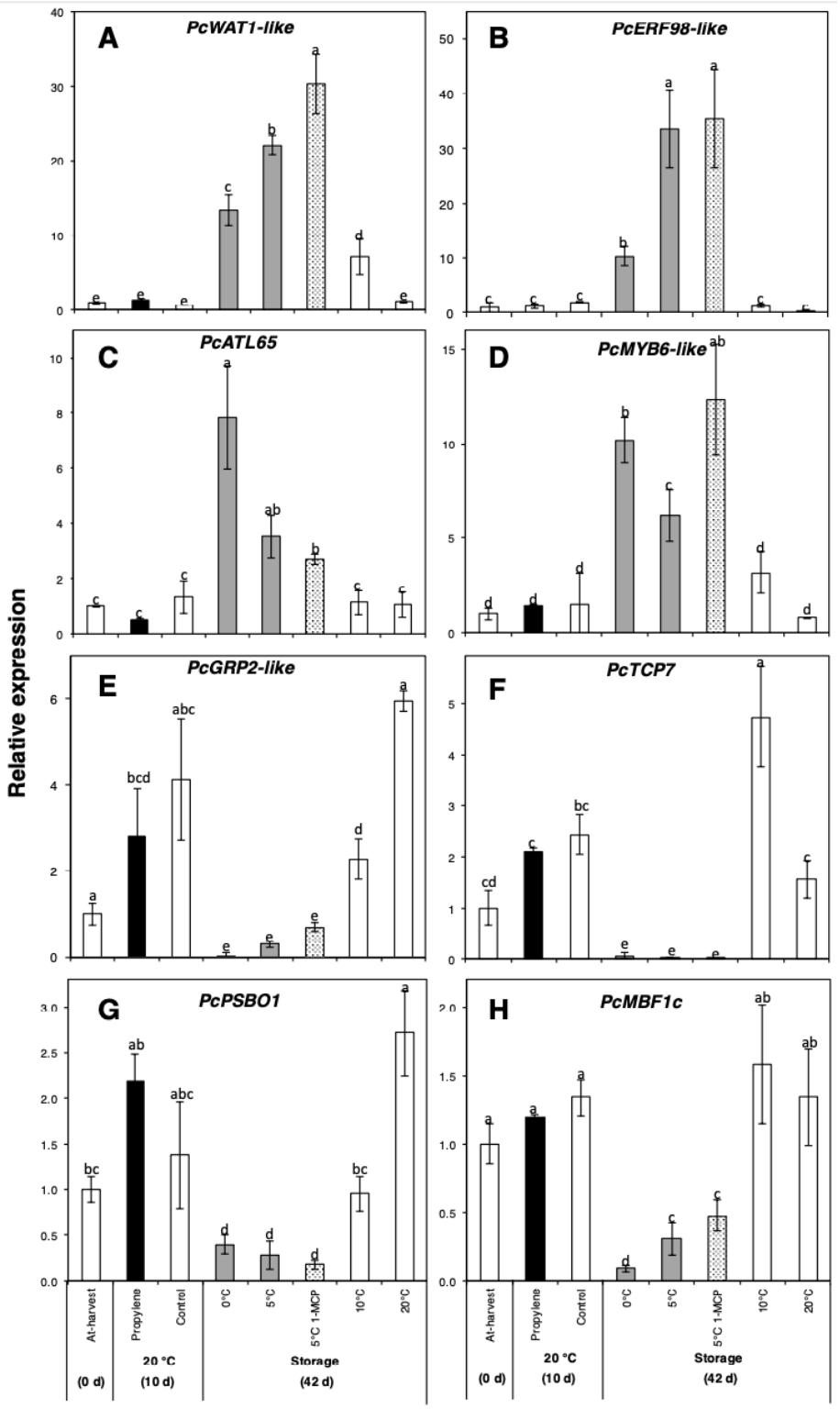


Fig. 3.9. Relative expression changes and RT-qPCR validation of selected low temperature-specific genes in 'Passe Crassane' pear fruit. Expression values were determined relative to *PcActin* with samples at harvest (0 d) set as 1. Values represent means (± SE) of three independent biological replications obtained from the 2016 experiment. Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

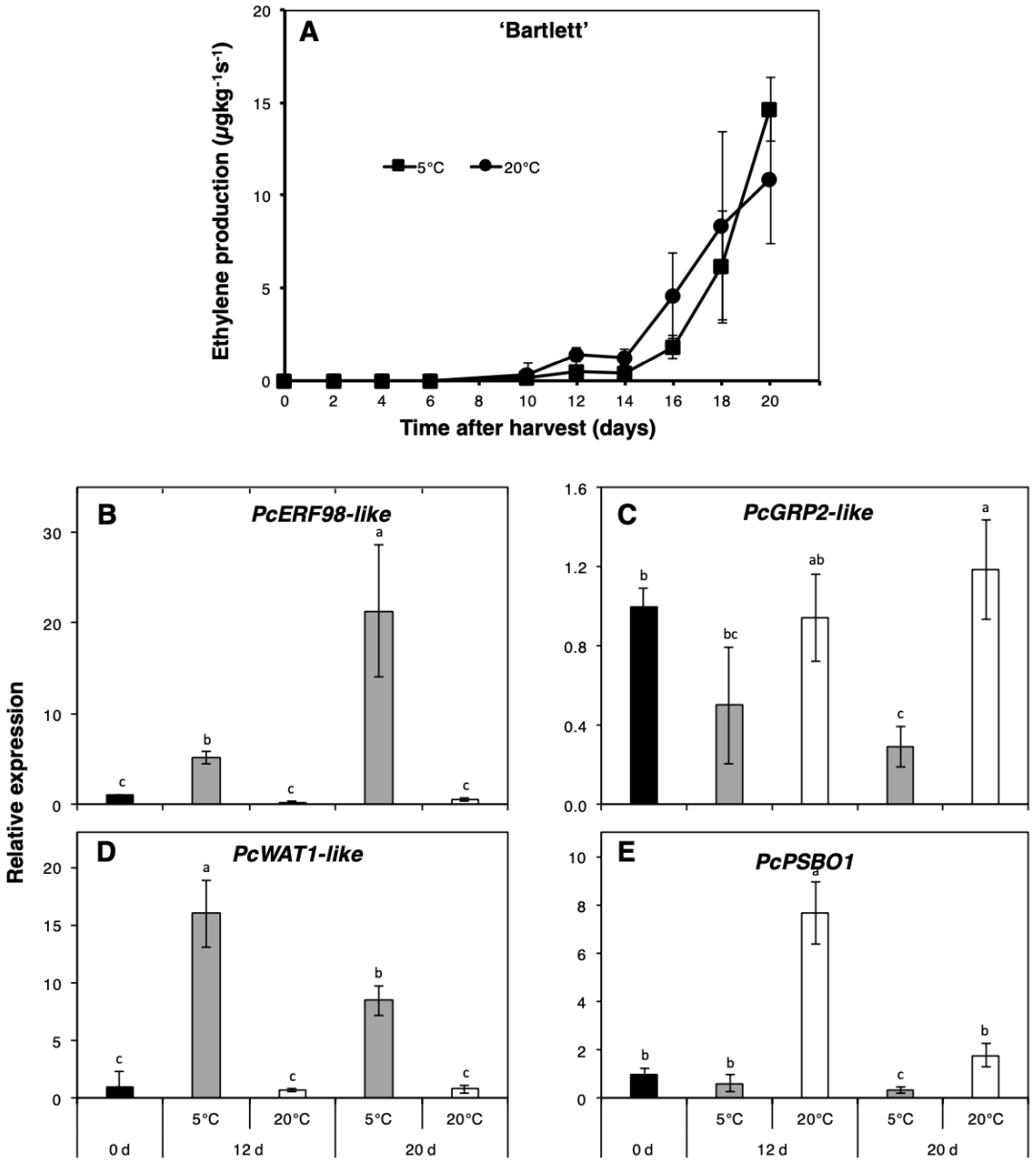


Fig. 3.10. Ethylene production rates (A) and relative expression changes of selected low temperature-specific genes (B–E) in 'Bartlett' pear fruit during storage at either 5 ºC or 20 ºC. Values represent means (± SE) of at least three independent biological replications. Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

3.3.9. Changes in the contents of phytohormones

We also quantified the levels of phytohormones in the flesh of 'Passe Crassane' fruit treated with propylene for 10 d, and after storage at various temperatures for 42 d using samples collected in 2016 (Table 3.1). Gibberellins (GA_1 and GA_4) and cytokinins (tZ and iP) were undetectable in fruit at all treatments. JA content decreased from the original 1.17 µg kg^{-1} at harvest to 0.37 µg kg^{-1} in propylene-treated fruit, 0.46 ng g^{-1} in fruit at 0 ºC and < 0.07 µg kg^{-1} at 5 ºC with or without 1-MCP treatment. There was also a significant decrease in JA content to 0.34 µg kg^{-1} in fruit at 20 ºC. However, we could not detect its active conjugate, JA-Ile, in fruit under all treatments. There was a great variation in IAA content as it decreased in propylene-treated fruit, increased at 0 ºC and 10 ºC, while it remained constant at 5 ºC and 20 ºC. ABA was detected at very high levels (386 µg kg^{-1}) in at-harvest fruit samples which doubled under propylene treatment (881 µg kg^{-1}), and almost tripled at 5 ºC (1117 µg kg^{-1}). Finally, SA increased in fruit stored at 0 ºC and 5 ºC by ~2 times the levels at harvest, while its levels were unchanged under the other treatments.

Table 3.1. Phytohormone levels in the flesh of 'Passe Crassane' fruit after continuous propylene treatment for 10 d, and storage at various temperatures for 42 d. Values are means (± SE) of three independent biological replications obtained in 2016. Different letters in the same row indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

	Concentration (µg kg^{-1})						
Phytohormone	**At-harvest**	**Propylene**	**0 ºC**	**5 ºC**	**5 ºC+1-MCP**	**10 ºC**	**20 ºC**
Indole-3-acetic acid	5.69^{ab} ± 2.26	1.84^{b} ±0.23	14.98^{a} ±4.70	4.16^{ab} ±1.61	4.48^{ab} ±0.71	10.44^{ab} ±3.63	5.10^{ab} ±0.64
Abscisic acid	386.25^{b} ±95.27	881.27^{ab} ±125.09	471.12^{b} ±113.15	1117.19^{a} ±239.28	402.27^{b} ±24.26	633.29^{ab} ±84.20	477.90^{b} ±86.57
Jasmonic acid (JA)	1.17^{a} ±0.74	0.37^{b} ±0.34	0.46^{b} ±0.26	0.05^{c} ±0.03	0.07^{c} ±0.02	0.86^{a} ±0.65	0.34^{b} ±0.14
Jasmonoyl-1-Isoleucine	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Gibberellins A_1	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Gibberellins A_4	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Salicylic acid	23.06^{b} ±0.57	18.61^{b} ±2.11	43.10^{a} ±12.28	47.27^{a} ±4.18	22.18^{b} ±3.06	24.73^{b} ±4.89	24.91^{b} ±7.74
trans-zeatin	n.d	n.d	n.d	n.d	n.d	n.d	n.d
N6-isopentenyl adenine	n.d	n.d	n.d	n.d	n.d	n.d	n.d

n.d: not detected

3.4. Discussion

European pear fruit require LT exposure for autocatalytic ethylene production and subsequent ripening. LT-mediated fruit ripening is associated with the induction of ethylene biosynthetic as well as ethylene-regulated genes (Lelièvre *et al*., 1997b; Hiwasa *et al*., 2003; El-Sharkawy *et al*., 2004). However, fundamental questions exist regarding LT-induced pathways during fruit ripening. What physio-molecular changes facilitate LT induction of ethylene production and subsequently, fruit ripening? Secondly, are there other molecular responses to LT exposure besides ethylene production and associated biosynthetic genes? In this work, we begin to address these questions by using integrated physiological and transcriptomic analyses to re-examine ethylene responses in 'Passe Crassane' fruit before and after chilling exposure.

The transcriptome analysis indicated that *ACS-like* genes did not respond to ethylene (propylene) in non-chilled fruit (Fig. 3.4), which, in all likelihood, should account for the little changes in endogenous ethylene production (Fig. 3.1A). Previous studies in tomatoes have shown that *ACS* genes regulate the rate-limiting step in ethylene biosynthesis (Yip *et al*., 1992; Wang *et al*., 2002). The observed concurrent increase in the expression of *PcACS1* and endogenous ethylene levels in LT-stored fruit (Fig. 3.2A, 3.6A) is compatible with this idea. On the contrary, four *ACO-like* genes including *PcACO1* were either induced by ethylene (propylene) in non-chilled fruit or LT exposure (Fig. 3.5, Fig. 3.6B), indicating their subordinate roles in ethylene biosynthesis. Notwithstanding, our finding that LT exposure induced ethylene and ethylene-biosynthetic genes agree with previous research in European pear fruit (Lelièvre *et al*., 1997b; Hiwasa *et al*., 2003; El-Sharkawy *et al*., 2004; Fonseca *et al*., 2005; Villalobos-Acuña and Mitcham, 2008), as well as other fruit such as apples (Lelièvre *et al*., 1995; Tian *et al*., 2002; Tacken *et al*., 2010).

3.4.1. Augmented ethylene responses after LT exposure in 'Passe Crassane' fruit

A distinct set of genes were regulated by propylene in non-chilled fruit, as well as by LT exposure (Fig. 3.3A). This group included four *ACO-like* genes that were up-regulated by either propylene or LT (Fig. 3.5). Additional members include several cell wall modification-associated genes that were induced in non-chilled propylene-treated fruit as well as in LT-stored fruit (Fig. 3.7A–D), which would account for the rapid softening in either treatments (Fig. 3.1B, 3.2B). Similar results have also been reported in previous works in European pear

fruit (Hiwasa *et al*., 2003; Fonseca *et al*., 2005). *Pcα-AMY* expression also increased in response to both propylene and LT exposure (Fig. 3.7E). It is undeniable that the above genes are under ethylene regulation in LT-stored fruit, based on the presence of substantial amounts of ethylene (Fig. 3.2A), and the effectiveness of 1-MCP treatment to suppress their expression. However, a closer look at the expression patterns of *PcACO1*, *PcPL18a*, *Pcβ-GAL2*, *PcEXP9* and *Pcα-AMY* shows that these transcripts accumulated at remarkably higher levels in fruit at 0 ºC and 5 ºC, compared to propylene-treated fruit (Fig. 3.6B, 3.7B–E). It is possible, therefore, that LT exposure triggers a change in the physio-molecular status of 'Passe Crassane' fruit, resulting in enhanced responses to ethylene.

3.4.2. Development of new ethylene responses after LT exposure

Another possible role of LT in European pear fruit ripening is to develop new responses to ethylene. This concept is supported by the observation that a distinct set of genes began to respond to ethylene exclusively after LT exposure (Fig. 3.3B, 3.8). A good example is our finding that *PcACS1* expression, together with autocatalytic ethylene production, did not respond to ethylene (propylene) in non-chilled fruit but only after storage at 0 ºC and 5 ºC (Fig. 3.2A, 3.6A). In this sense, LT exposure appears to facilitate ethylene regulation of its own biosynthesis (positive feedback system); a typical feature of all climacteric fruit (Kende, 1993).

Our study also observed that *Pc2OGD* expression became ethylene-responsive after LT exposure (Fig. 3.8A). The 2OGD superfamily comprises genes with a wide range of functions (Farrow and Facchini, 2014); some bacteria such as *Pseudomonas syringae* and *Penicillium digitatum* are known to utilize this pathway for ethylene production (Johansson *et al*., 2014; Zhang *et al*., 2017). In 'Passe Crassane' fruit, it is unclear whether *Pc2OGD* is involved in ethylene biosynthesis, but its expression pattern supports the idea that new ethylene responses develop after LT exposure.

Ethylene receptors are negative regulators of the signalling pathway, and ethylene binding effectively switches off their inhibitory effect (Tieman *et al*., 2000). Therefore, it is plausible that an increase in ethylene production would provoke a concomitant increase in receptor levels to counter the ethylene effect. In this study, our finding that *PcETR2* expression concurrently increased with increased ethylene levels in LT-stored fruit agrees with previous research in 'Passe Crassane' pears (El-Sharkawy *et al*., 2003). This would concur with the model that the

primary role of ethylene receptors is to temper ethylene responses (Klee, 2002; Ireland *et al*., 2014).

Prior studies in apple fruit have demonstrated the existence of variations in ethylene sensitivity among cell wall-modifying genes (Ireland *et al*., 2014), and that this may be influenced by cold (Tacken *et al*., 2010). This is consistent with our finding that some 'Passe Crassane' genes such as *PcEG3*, *PcEXPA1-like* and *PcPL18b* exhibited little response to ethylene (propylene) in non-chilled fruit but showed a strong ethylene-dependent expression pattern after LT exposure (Fig. 3.8C–E). Therefore, it is more likely that the transcriptional adjustments brought about by LT also function to facilitate the responses of the above cell wall-modifying genes to ethylene.

A notable functional group among genes responding to ethylene after LT exposure included those encoding TFs such as *PcMADS2* and *PcBZR1* (Fig. 3.8G, H). Various MADS-box TFs such as RIN are known to directly regulate ethylene biosynthetic genes and ripening-related genes (Vrebalov *et al*., 2002). However, ethylene was also shown to regulate the expression of *RIN* (Fujisawa *et al*., 2013), as well as several *MaMADS* in bananas (Elitzur *et al*., 2010). Recently, two genes encoding brassinosteroid pathway TFs, *MaBZR1/2*, were shown to be under ethylene regulation in bananas (Guo *et al*., 2019). The present work however suggests that the ability of ethylene to regulate *PcMADS2* and *PcBZR1* expression is facilitated by cold signalling.

3.4.3. Potential functions of LT-specific genes in European pear fruit ripening

The present study demonstrates that LT-mediated fruit ripening in European pears is an aggregate of autocatalytic ethylene induction, amplification of existing ethylene responses and development of new ethylene responses. These are most likely to result from a physio-molecular shift in the European pear fruit during chilling. While the nature of this physio-molecular shift remains unclear, possible candidates include genes that showed LT-specific ethylene-independent expression patterns in 'Passe Crassane' fruit (Fig. 3.9), and even in 'Bartlett' pears (Fig. 3.10). Genes in this category are likely to modulate both ethylene biosynthetic and responsive genes to enhance fruit ripening.

The importance of ERF genes in ethylene biosynthesis has been well documented. In apples, it was shown that MdERF2 suppresses *MdACS1* expression while MdERF3 promotes its expression (Li *et al*., 2016; 2017). Zhang *et al*. (2009) also demonstrated that LeERF2 modulates ethylene biosynthesis in tomatoes via interaction with ethylene biosynthetic genes. In the present work, therefore, the LT-specific expression of *PcERF98-like* in European pear fruit (Fig. 3.9B) suggests its possible role in modulating ethylene biosynthetic such as *PcACS1* and *PcACO1*, as well as ethylene-regulated genes to promote fruit ripening.

Zinc finger TFs have been shown to regulate diverse biological processes in plants (Englbrecht *et al*., 2004). In tomatoes, SlZFP2 was shown to interact with COLOURLESS NON-RIPENING (CNR) to indirectly regulate ethylene biosynthesis (Weng *et al*., 2015). Given that *PcATL65* and *PcGRP2-like* exhibited LT-specific expression patterns in European pear fruit (Fig. 3.9C, E; 3.10C), it is possible that they might play a role in modulating LT-induced changes in ethylene production and ethylene responsiveness.

Plant MYBs also play significant roles in fruit ripening regulation. Previous studies in apples have shown that *MdMYBA* and *MdMYB10* promote anthocyanin biosynthesis and red coloration in the apple skin (Ban *et al*., 2007; Espley *et al*., 2007). Recently, MaMYB3 was shown to regulate banana fruit ripening by modulating starch degradation (Fan *et al*., 2018). In European pears, *PcMYB6-like* was exclusively regulated by cold (Fig. 3.9D, 3.10A), suggesting its possible role in autocatalytic ethylene production and increased ethylene responsiveness following LT exposure.

Various TCP family TFs including MaTCP5, MaTC19 and MaTCP20, have been recently shown to regulate banana fruit ripening by modulating the transcription of *MaXTH10/11* (Song *et al*., 2018). Guo *et al*. (2018) demonstrated that *PpTCP.A2* regulates ethylene biosynthesis in peach fruit, likely via transcriptional repression of *PpACS1*. We demonstrate the LT-specific expression of *PcTCP7* in European pears (Fig. 3.9F), further suggesting a mechanism by which ethylene-biosynthetic and ethylene-regulated genes are regulated during cold treatment.

In Arabidopsis, the transcriptional coactivator, MBF1c, was shown to function upstream of salicylic acid, trehalose, ethylene, and pathogenesis-related protein 1 during heat stress (Suzuki *et al*., 2008). Some members of MBF1c family also regulate fruit ripening in tomatoes (Sanchez-Ballesta *et al*., 2007). In this study, based on the LT-specific expression of *PcMBF1c*

in ‘Passe Crassane’ fruit (Fig. 3.9H), it appears that this gene might also play a role cold-induced changes in ethylene production and ethylene responsiveness.

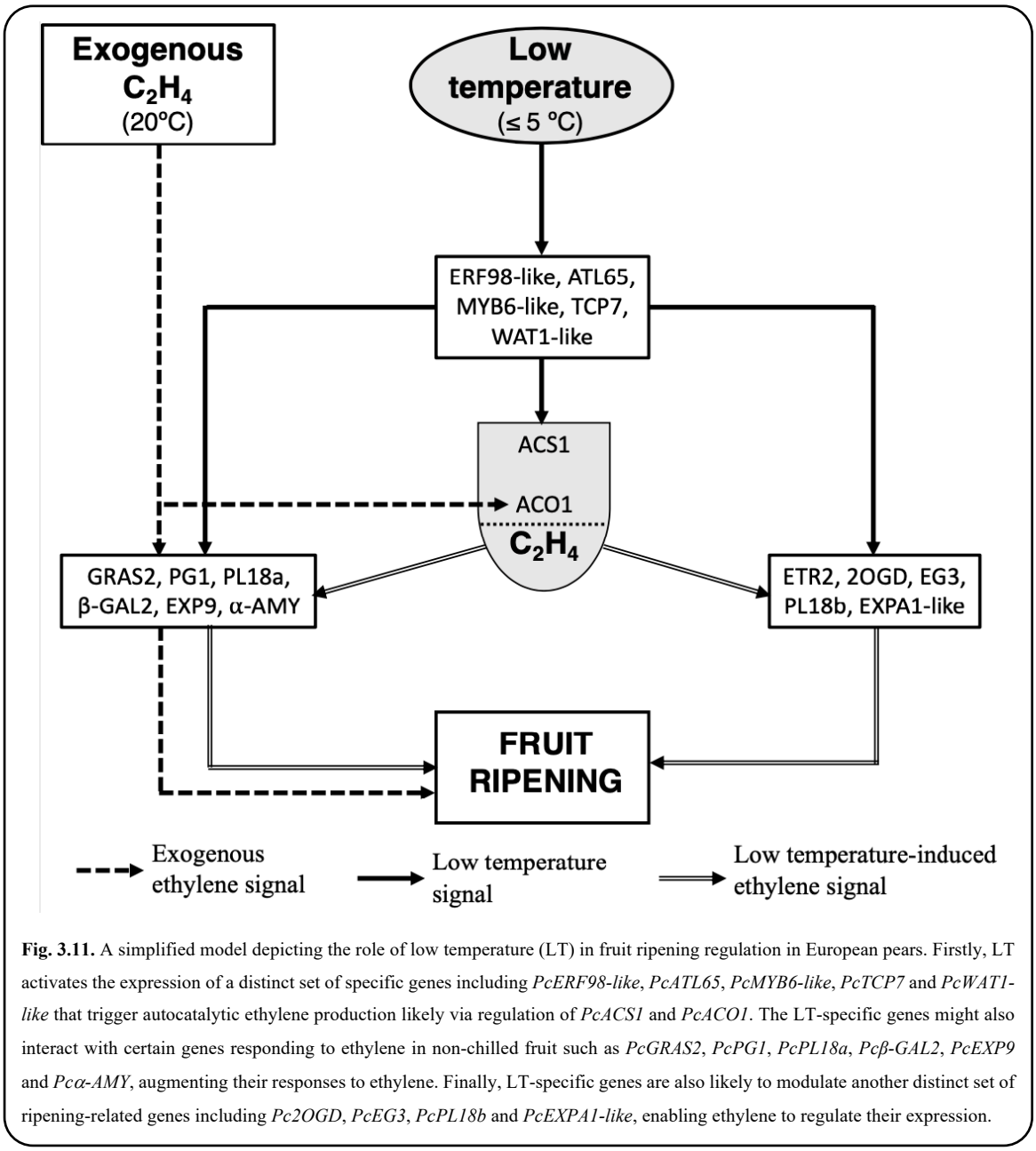


Fig. 3.11. A simplified model depicting the role of low temperature (LT) in fruit ripening regulation in European pears. Firstly, LT activates the expression of a distinct set of specific genes including *PcERF98-like*, *PcATL65*, *PcMYB6-like*, *PcTCP7* and *PcWAT1-like* that trigger autocatalytic ethylene production likely via regulation of *PcACS1* and *PcACO1*. The LT-specific genes might also interact with certain genes responding to ethylene in non-chilled fruit such as *PcGRAS2*, *PcPG1*, *PcPL18a*, *Pcβ-GAL2*, *PcEXP9* and *Pcα-AMY*, augmenting their responses to ethylene. Finally, LT-specific genes are also likely to modulate another distinct set of ripening-related genes including *Pc2OGD*, *PcEG3*, *PcPL18b* and *PcEXPA1-like*, enabling ethylene to regulate their expression.

Hormonal interplay is considered an important aspect of fruit ripening regulation. Auxins regulate various genes associated with ethylene biosynthesis and response in peaches (Trainotti *et al*., 2007; Tatsuki *et al*., 2013). ABA has been shown to facilitate ethylene biosynthesis and responses in tomato via regulation of *LeACS4*, *LeACO1* and *LeERT6* as well as some ripening-related regulators such as *RIN* and *CNR* (Mou *et al*., 2016). In this study, there was a great variation in phytohormone contents during ripening in ‘Passe Crassane’ fruit (Table 3.1). This is partly due to their presence in extremely low concentrations and partly because they vary

greatly among tissues (Cao *et al*., 2016), making their quantification difficult. Nonetheless, ABA content consistently increased in the presence of ethylene/propylene before and after chilling, suggesting that it might work together with ethylene to promote 'Passe Crassane' fruit ripening. Interestingly, *PcWAT1-like*, whose homolog in Arabidopsis was shown to encode a vacuolar auxin transporter (Ranocha *et al*., 2013), exhibited a consistent LT-specific expression pattern in European pears (Fig. 3.9A, 3.10D). Considering that IAA levels did not change significantly during 'Passe Crassane' pear fruit ripening (Table 3.1), it would be intriguing to explore whether auxin transport (possibly by *PcWAT-like*) also mediates LT-triggered ripening processes.

3.5. Conclusion

While there is little doubt that LT induces, and/or promotes fruit ripening in most European pear cultivars (Lelièvre *et al*., 1997b; El-Sharkawy *et al*., 2004; Villalobos-Acuña and Mitcham, 2008), the distinctions between the roles of LT-induced ethylene and LT alone remain unclear. Here, we propose a model (Fig. 3.11), in which LT-specific genes modulate fruit ripening-associated processes on three fronts: (i) initiation of autocatalytic ethylene production (LT-induced ethylene) by modulating ethylene biosynthetic genes; (ii) augmenting already existing ethylene responses; and (iii) enabling a new set of genes to respond to LT-induced ethylene. This work lays a foundation for elaborating the precise role of LT signal transduction pathway in fruit ripening regulation.

CHAPTER 4

Regulation of peel degreening in citrus fruit by low temperature

4.1. Ethylene-independent modulation of natural peel degreening in lemon (*Citrus limon* L.) fruit by low temperature

Abstract

Peel degreening is an important aspect of fruit ripening in many citrus fruit, and earlier studies have shown that it can be advanced either by ethylene treatment or during low temperature storage. However, the important regulators and pathways involved in natural peel degreening remain largely unknown. To understand how natural peel degreening is regulated in lemon (*Citrus limon* L.) fruit, flavedo transcriptome and physiochemical changes in response to either ethylene treatment or low temperature were studied. Ethylene treatment induced rapid peel degreening which was strongly inhibited by the ethylene antagonist, 1-methylcyclopropene (1-MCP). Compared with 25 ºC, moderately low temperatures (5 ºC, 10 ºC, 15 ºC and 20 ºC) also triggered peel degreening. Surprisingly, repeated 1-MCP treatments failed to inhibit the peel degreening induced by low temperature. Transcriptome analysis revealed that low temperature and ethylene independently regulated genes associated with chlorophyll degradation, carotenoid metabolism, photosystem proteins, phytohormone biosynthesis and signalling, and transcription factors. On-tree peel degreening occurred along with environmental temperature drops, and it coincided with the differential expression of low temperature-regulated genes. In contrast, genes that were uniquely regulated by ethylene showed no significant expression changes during on-tree peel degreening. Based on these findings, we hypothesize that low temperature plays a prominent role in regulating natural peel degreening independently of ethylene in citrus fruit.

4.1.1. Introduction

Fruit ripening is a multifaceted process comprising various physiochemical and structural changes such as softening, starch degradation to sugars, colour development and aroma volatile production (Cherian *et al*., 2014; Seymour and Granell, 2014). In citrus fruit, colour development, commonly known as peel degreening, is a critical part of fruit ripening which is characterized by peel colour change from green to yellow/red/orange (Iglesias *et al*., 2007). Peel degreening is an important aspect for marketability of citrus fruit (Porat, 2008), and thus there is wide interest in unravelling the fundamental regulatory mechanisms involved.

There are two main pathways that have been linked to citrus peel degreening. One is chlorophyll degradation, which firstly involves dephytilation of chlorophyll molecules by chlorophyllase (CLH) and pheophytinase (PPH) followed by removal of the central Mg atom by Mg-dechelatase to form pheophorbide. Pheophorbide is then converted to red chlorophyll catabolites (RCC) by pheophorbide a oxidase (PaO), and RCC is reduced to colourless compounds by RCC reductase (RCCR) (Hörtensteiner, 2006; Shimoda *et al*., 2016; Yin *et al*., 2016). The other is carotenoid biosynthesis which starts with the condensation of two geranylgeranyl pyrophosphate (GGPP) molecules by phytoene synthase (PSY) to form phytoene. Phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) successively convert phytoene to lycopene, which is then converted to either α-carotene or β-carotene by lycopene ε-cyclase (LCYe) and lycopene β-cyclase (LCYb) respectively. α-carotene is later converted to lutein via sequential hydroxylation by ε-ring hydroxylase and β-ring hydroxylase (CHYb), whereas β-carotene is converted to zeaxanthin via β-cryptoxanthin by CHYb (Cunningham *et al*., 1996; Ohmiya *et al*., 2019). Genes encoding various enzymes for the main steps of chlorophyll degradation and carotenoid metabolism have been isolated and functionally characterized (Rodrigo *et al*., 2013).

The phytohormone ethylene has been routinely used for commercial degreening in citrus fruit (Purvis and Barmore, 1981; Porat, 2008; Mayuoni *et al*., 2011). Exogenous ethylene application was shown to transcriptionally modulate both chlorophyll degradation and carotenoid metabolism (Rodrigo and Zacarias, 2007; Shemer *et al*., 2008; Yin *et al*., 2016). Transcription factors (TF) that may be involved in ethylene-induced peel degreening have also been identified and characterized (Yin *et al*., 2016). Nevertheless, it remains unclear whether ethylene plays a role during natural degreening since citrus fruit are non-climacteric and the amounts of ethylene produced are minute (Eaks, 1970; Sawamura, 1981; Katz *et al*., 2004).

Temperature has a large impact on a wide range of plant growth and developmental processes, including fruit ripening and maturation. Low temperature is thought to slow most cell metabolic activities, and hence it is the major postharvest technology used to delay fruit ripening and senescence (McGlasson *et al*., 1979; Hardenburg *et al*., 1986). However, promotion of fruit ripening by low temperature has been described in various fruit species such as kiwifruit (Kim *et al*., 1999; Mworia *et al*., 2012; Asiche *et al*., 2017; Mitalo *et al*., 2018a), European pears (El-Sharkawy *et al*., 2003; Nham *et al*., 2017), and apples (Tacken *et al*., 2010).

Recently, transcriptome studies have suggested that low temperature-specific genes might have regulatory roles during fruit ripening in kiwifruit (Asiche *et al*., 2018; Mitalo *et al*., 2018a; Mitalo *et al*., 2018b; Mitalo *et al*., 2019a; Mitalo *et al*., 2019b) and European pears (Mitalo *et al*., 2019c).

Citrus fruit are among the species where low temperature has been linked to fruit ripening, especially peel degreening. Typically, as most citrus fruit mature on the tree, the seasonal temperature drops. Multiple studies have thus demonstrated that cold periods below 13 ºC are required to stimulate on-tree fruit colour development (Manera *et al*., 2012; Manera *et al*., 2013; Rodrigo *et al*., 2013; Conesa *et al*., 2019). During storage, low/intermediate temperatures (6–15 ºC) have also been shown to promote peel degreening (Matsumoto *et al*., 2009; Van Wyk *et al*., 2009; Zhu *et al*., 2011; Carmona *et al*., 2012a; Tao *et al*., 2012). Natural peel degreening in citrus fruit has often been attributed to ethylene, on the assumption that the basal system I ethylene levels are physiologically active (Goldschmidt *et al*., 1993; Carmona *et al*., 2012b). Whether the peel colour changes during on-tree maturation and low temperature storage are caused by basal ethylene, low temperature and/or a synergistic effect of ethylene and low temperature, or because of another mechanism is still not yet clear.

Here, we examined the peel degreening behaviour of lemon fruit in response to exogenous ethylene and different storage temperatures (5 ºC, 10 ºC, 15 ºC, 20 ºC, and 25 ºC). We found that both ethylene treatment and moderately low temperatures (5 ºC, 10 ºC, 15 ºC and 20 ºC; hereinafter referred to as low temperature) promoted peel degreening. Further, we explored the role of ethylene in low temperature-triggered peel degreening using repeated treatments with 1-methylcyclopropene (1-MCP), a well-known ethylene antagonist (Watkins, 2006; Sisler and Serek, 1997). We found that 1-MCP treatments did not inhibit the accelerated peel colour changes induced by low temperature. Further transcriptome analysis revealed that ethylene and low temperature independently regulated distinct gene sets in the flavedo of lemon fruit. On-tree peel degreening also coincided with a decrease in minimum environmental temperatures and differential expression of low temperature-regulated genes, whereas ethylene-specific genes showed no significant expression changes. These results suggested that low temperature might transcriptionally modulate peel degreening independently of basal endogenous ethylene.

4.1.2. Materials and methods

4.1.2.1. Plant material and treatments

Lemon fruit (*C. limon* L. cv. 'Allen Eureka') grown under standard cultural practices were collected in 2018 from a commercial orchard in Takamatsu (Kagawa, Japan). Sampling was from seven harvests during fruit development: 3 Sep., 27 Sep., 12 Oct., 30 Oct., 14 Nov., 29 Nov., and 13 Dec., corresponding to 171, 196, 211, 230, 246, 261 and 276 days after full bloom (DAFB), respectively. To characterize postharvest ethylene effect, lemons (196 DAFB) were divided into four lots of 20 fruit each. The first set of fruit contained non-treated fruit (control), while the second set were treated with 1-MCP (2 μLL^{-1}) for 12 h. The third set of fruit were continuously treated with ethylene (100 μLL^{-1}), while the fourth set were initially treated with 1-MCP (2 μLL^{-1}) for 12 h followed by continuous ethylene (100 μLL^{-1}) treatment. 1-MCP was released by dissolving SmartFresh™ powder (AgroFresh, PA, United States) in water. All treatments were carried out at 25 ºC for up to 8 d. For postharvest storage tests, lemons (196 DAFB) were divided into five lots of 40 fruit each, and stored at either 5 ºC, 10 ºC, 15 ºC, 20 ºC or 25 ºC for up to 42 d. Additionally, three separate sets (40 fruit each) were stored at either 5 ºC, 15 ºC or 25 ºC with repeated (twice a week) 12 h 1-MCP treatments. Fruit peel (flavedo) was sampled, frozen in liquid nitrogen and stored at -80 ºC for future analysis, each sample containing three biological replicates.

4.1.2.2. Citrus colour index (CCI) determination

The *L*, *a* and *b* Hunter lab parameters were measured on four even equatorial sites on the fruit surface using a Minolta CR-200B chromameter (Konica Minolta, Tokyo, Japan). CCI values were presented as the results of 1000•*a*/*L*•*b* transformation, expressed as a mean of five fruit.

4.1.2.3. Determination of chlorophyll and carotenoid content

Chlorophylls were extracted and quantified in triplicate according to the procedure by Rodrigo *et al*. (2003) with slight modifications. Chlorophylls were extracted in 80 % acetone and appropriate dilutions were used to quantify absorbance at 646.8 nm and 663.2 nm. Chlorophyll content was calculated from these measurements using Lichtenthaler and Wellburn equations (Wellburn, 1994). Extraction and quantification of carotenoids were conducted in triplicate according to the procedures by Kato *et al*. (2004) and Matsumoto *et al*. (2007) with slight modifications. Briefly, carotenoids were successively extracted with 40 % methanol and

diethyl ether/methanol (containing 0.1 % BHT). After saponification with methanolic potassium hydroxide, the organic layer of the extracts was vacuum-dried and analysed by HPLC. The HPLC analysis was carried out on an Extrema LC-4000 system (Jasco, Tokyo, Japan) equipped with a photo diode-array detector and autosampler. Samples were analysed on a Develosil C30-UG column (3 µm, 150 x 4.6 mm, Nomura Chemicals, Aichi, Japan) set at 20 ºC and 0.5 mL/min flow rate. The UV-Vis spectra were obtained between 250 and 550 nm, and chromatograms were processed at 450 nm. Carotenoid quantifications were based on standard curves generated using authentic standards.

4.1.2.4. Phytohormone measurements

Phytohormone extraction and analysis were performed according to the method described by Gupta *et al*. (2017), using deuterium-labelled internal standards for indole-3-acetic acid (IAA), abscisic acid (ABA), jasmonic acid (JA), gibberellins (GAs), t*rans*-zeatin (tZ), N6-isopentenyladenine (iP) and salicylic acid (SA), and ^{13}C-labelled jasmonoyl-*L*-isoleucine (JA-Ile). Eluted fractions were analysed on an Agilent 1260-6410 Triple Quad LC/MS system equipped with a ZOR-BAX Eclipse XDB-C18 column (Agilent Technologies, CA, USA). Liquid chromatography conditions are described in Appendix 4, while the multiple-reaction-monitoring mode of the tandem quadrupole mass spectrometer and precursor-product ion transitions for each compound are listed in Appendix 5.

4.1.2.5. RNA-seq and differential gene expression analysis

Total RNA was extracted in triplicate from the flavedo of ethylene-treated and control (non-treated) lemon fruit after 4 d, as well as fruit after 28 d of storage at 5 ºC, 15 ºC and 25 ºC. Illumina paired-end libraries were constructed using NEBNext® Ultra™ RNA Library Prep Kit for Illumina (New England Biolabs, MA, USA), before being sequenced using Illumina HiSeq 2500 platform (Hokkaido System Co. Ltd., Japan). Trimming was done to obtain ≥ 10 million paired reads per sample, and the reads were mapped to the reference *Citrus clementina* Genome v1.0 (Wu *et al*., 2014). Gene expression levels were calculated using the reads per kilobase per million (RPKM) method and differentially expressed genes (DEGs) were identified using the false discovery rates (FDR) analysis (Robinson *et al*., 2010). DEG selection was based on two criteria: (i) genes with RPKM ≥ 3.0 and FDR ≤ 0.001, and (ii) fold change ≥ 3.0 in average RPKM for ethylene vs. control, 5 ºC vs. 25 ºC and/or 15 ºC vs. 25 ºC. For co-expression analysis, the WGCNA method (Zhang and Horvath, 2005) was used to generate modules of

highly correlated genes based on the RNA-seq expression data. Gene modules were identified by implementing the WGCNA package in R (Langfelder and Horvath, 2008). The soft-thresholding power and tree-cut parameters used for the WGCNA analysis were 12 and 0.15, respectively.

4.1.2.6. Reverse-transcriptase quantitative PCR (RT-qPCR)

Total RNA was extracted from the flavedo of fruit at harvest (0 d), after 4 d for ethylene, 1-MCP + ethylene, and control groups, and after 28 d storage at 5 ºC, 10 ºC, 15 ºC, 20 ºC and 25 ºC. Total RNA was also extracted from on-tree fruit samples at each of the specified sampling dates. DNase I (Nippon Gene, Tokyo, Japan) treatment followed by clean-up using FavorPrep after Tri-Reagent RNA Clean-up Kit (Favorgen Biotech. Co., Ping-Tung, Taiwan) were carried out to remove genomic DNA contamination from the extracted RNA. For all treatments, 2.4 µg of clean RNA was reverse transcribed to cDNA using Takara RNA PCR kit (Takara, Kyoto, Japan). Gene-specific primers (Appendix 8) were designed using Primer3 software (version 0.4.0, http://bioinfo.ut.ee/primer3-0.4.0/). Gene expression of three biological replicates was examined on MYiQ Single-Color Reverse Transcriptase-Quantitative PCR Detection System (Bio-Rad, Hercules, CA, USA) using TB Green™ Premix ExTaq™ II (Takara, Kyoto, Japan). *ClActin* (*Ciclev10025866m.g*) was used as the housekeeping gene after examining its constitutive expression pattern from the RNA-seq results. Relative expression values were calculated using 196 DAFB (0 d) fruit.

4.1.2.7. Statistical analysis

Data presented in this study were subjected to statistical analysis using R version 3.4.0 software package (R Project). ANOVA followed by post-hoc Tukey's test ($P < 0.05$) were used to detect statistical differences in CCI, pigment and phytohormone contents, and gene expression.

4.1.3. Results

4.1.3.1. Ethylene-induced peel degreening

To validate the role of ethylene in citrus peel degreening, detached lemon fruit were continuously treated with ethylene and/or its antagonist, 1-MCP. As expected, peel colour of ethylene-treated fruit started to change from green to yellow after 2 d, attaining a full yellow colour after 8 d (Fig. 4.1.1A). This change was numerically indicated by a rapid increase in

CCI from -14.2 at 0 d to -1.8 after 8 d. Notably, fruit pre-treated with 1-MCP followed by continuous ethylene treatment retained their greenish peel colour and CCI showed no significant changes throughout the experimental period. These findings demonstrated that 1-MCP pre-treatment rendered the fruit insensitive to ethylene, effectively inhibiting ethylene action on the peel colour.

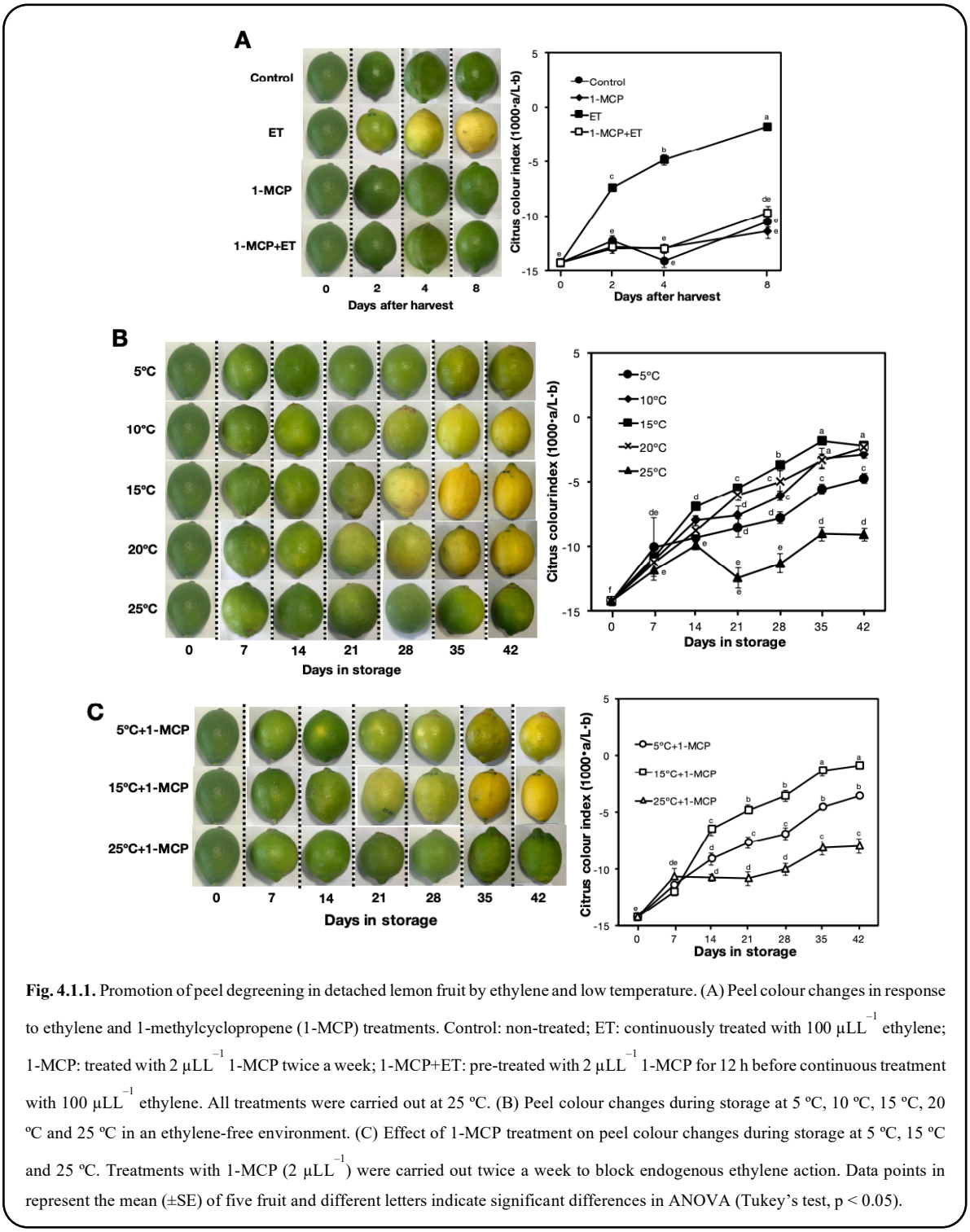


Fig. 4.1.1. Promotion of peel degreening in detached lemon fruit by ethylene and low temperature. (A) Peel colour changes in response to ethylene and 1-methylcyclopropene (1-MCP) treatments. Control: non-treated; ET: continuously treated with 100 μLL^{-1} ethylene; 1-MCP: treated with 2 μLL^{-1} 1-MCP twice a week; 1-MCP+ET: pre-treated with 2 μLL^{-1} 1-MCP for 12 h before continuous treatment with 100 μLL^{-1} ethylene. All treatments were carried out at 25 ºC. (B) Peel colour changes during storage at 5 ºC, 10 ºC, 15 ºC, 20 ºC and 25 ºC in an ethylene-free environment. (C) Effect of 1-MCP treatment on peel colour changes during storage at 5 ºC, 15 ºC and 25 ºC. Treatments with 1-MCP (2 μLL^{-1}) were carried out twice a week to block endogenous ethylene action. Data points in represent the mean (±SE) of five fruit and different letters indicate significant differences in ANOVA (Tukey's test, $p < 0.05$).

4.1.3.2. Peel degreening behaviour at different storage temperatures and the effect of 1-MCP

Peel colour changes in detached lemon fruit were also investigated during storage at different temperatures. As shown in Fig. 4.1.1B, peel colour of fruit at 5 ºC, 10 ºC, 15 ºC and 20 ºC gradually changed from green to yellow with a concomitant increase in CCI to about -2.3 after 28–42 d. Peel degreening was more pronounced at 15 ºC followed by 10 ºC and 20 ºC, than 5 ºC at which fruit retained appreciable greenish colour even after 42 d. In contrast, fruit at 25 ºC retained their greenish peel colour and the CCI changes were minimal throughout the storage period. These observations indicated that moderately low storage temperatures promoted the peel degreening process in lemon fruit.

To determine whether the basal levels of system I ethylene played a role in the observed low temperature-modulated peel degreening, we treated lemon fruit repeatedly with 1-MCP. Surprisingly, comparable peel degreening was observed in fruit at 5 ºC and 15 ºC but not at 25 ºC, notwithstanding the repeated 1-MCP treatments (Fig. 4.1.1C). Together, these findings suggested that low temperature may promote peel colour changes in lemon fruit independently of ethylene.

4.1.3.3. Differential expression analysis in lemon fruit flavedo

Overview of the transcriptome changes

To gain a deeper insight into the mechanisms of low temperature promotion of peel degreening, we conducted a comprehensive transcriptome analysis to compare low temperature-induced responses with those activated by ethylene. Ethylene-induced responses were captured by examining 4 d ethylene-treated and non-treated (control) flavedo samples. To cover low temperature-triggered responses, samples obtained after 28 d of storage at 5 ºC and 15 ºC were examined against those at 25 ºC.

RNA-seq analysis resulted in the identification of 3105 DEGs (q-value < 0.001), which responded to either ethylene or low temperature (Fig. 4.1.2). Ethylene had the largest share, influencing 2329 DEGs as opposed to 5 ºC and 15 ºC that influenced 1634 and 597 DEGs, respectively (Fig. 4.1.2A). In all treatments, the number of downregulated DEGs was higher than that of upregulated genes. Clustering analysis classified the DEGs into distinct groups that were regulated by either ethylene, 5 ºC and/or 15 ºC (Fig. 4.1.2B). Ethylene treatment

exclusively upregulated and downregulated 592 and 700 genes, respectively (Fig. 4.1.2C, D). Likewise, an aggregate of 337 and 439 genes were exclusively upregulated and downregulated, respectively by 5 ºC and 15 ºC. The remaining DEGs (420 upregulated and 617 downregulated) were jointly influenced by either ethylene, 5 ºC and/or 15 ºC. Altogether, identified DEGs could be pooled into three distinct groups. The first group comprised ethylene-specific genes, the second group included low temperature-specific genes while the third group consisted of genes regulated by either ethylene or low temperature.

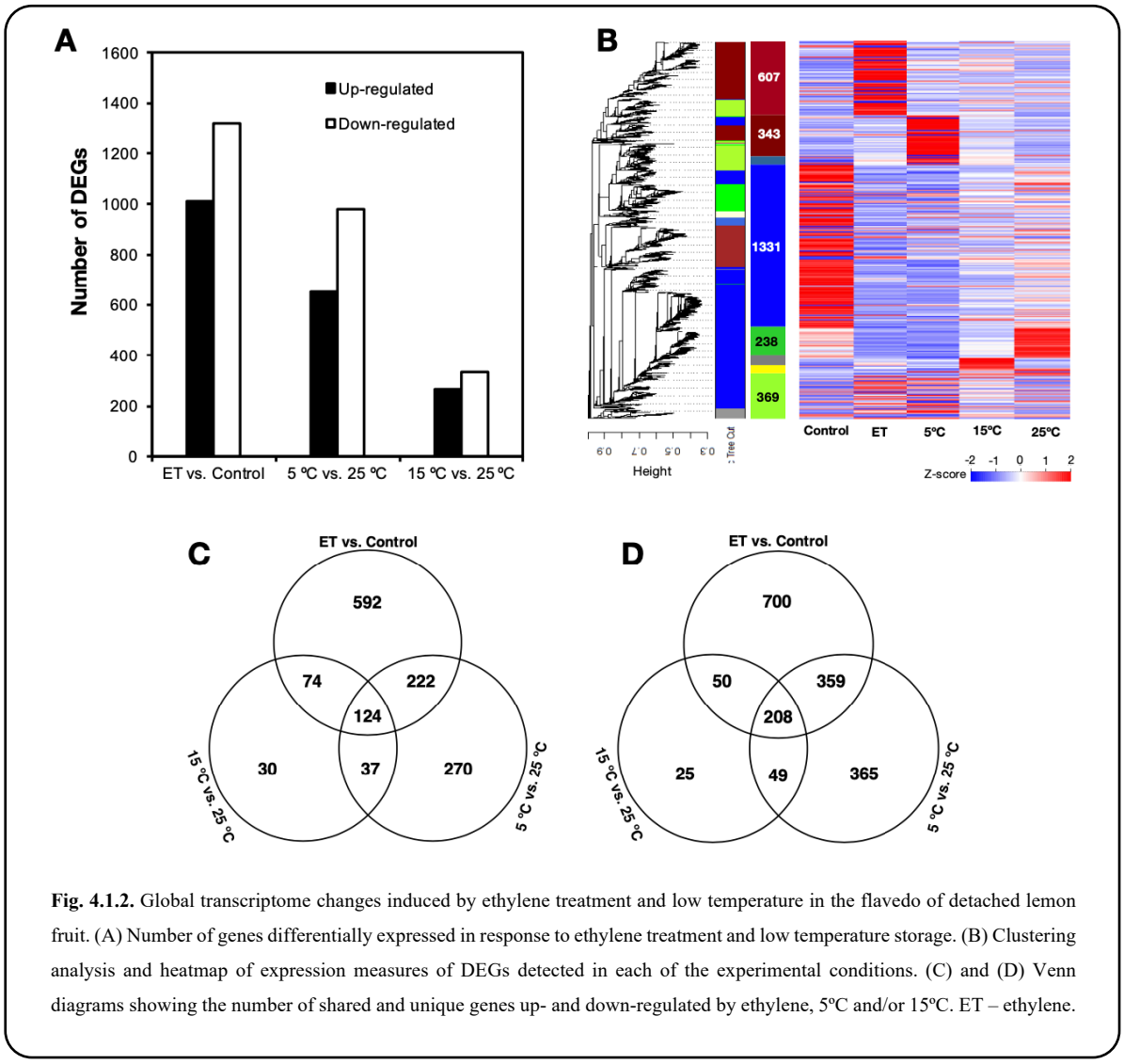


Fig. 4.1.2. Global transcriptome changes induced by ethylene treatment and low temperature in the flavedo of detached lemon fruit. (A) Number of genes differentially expressed in response to ethylene treatment and low temperature storage. (B) Clustering analysis and heatmap of expression measures of DEGs detected in each of the experimental conditions. (C) and (D) Venn diagrams showing the number of shared and unique genes up- and down-regulated by ethylene, 5ºC and/or 15ºC. ET – ethylene.

Chlorophyll metabolism and associated transcripts

Peel degreening is primarily caused by the degradation of green-coloured chlorophyll pigments to colourless non-fluorescent derivatives (Hortensteiner, 2006). Upon ethylene treatment for 4 d, peel chlorophyll a and b content drastically decreased from about 50 µg g^{-1} at harvest to merely 11 µg g^{-1} (Fig. 4.1.3A). However, ethylene treatment failed to induce chlorophyll

reduction in fruit pre-treated with 1-MCP which was in close agreement with the observed colour changes (Fig. 4.1.1A). During storage, peel chlorophyll content also decreased in fruit at moderately low temperatures (5 ºC, 10 ºC, 15 ºC and 20 ºC), whereas they were maintained at high levels in fruit at 25 ºC. It is noteworthy that peel chlorophyll content also decreased in fruit at 5 ºC and 15 ºC despite repeated treatments with 1-MCP.

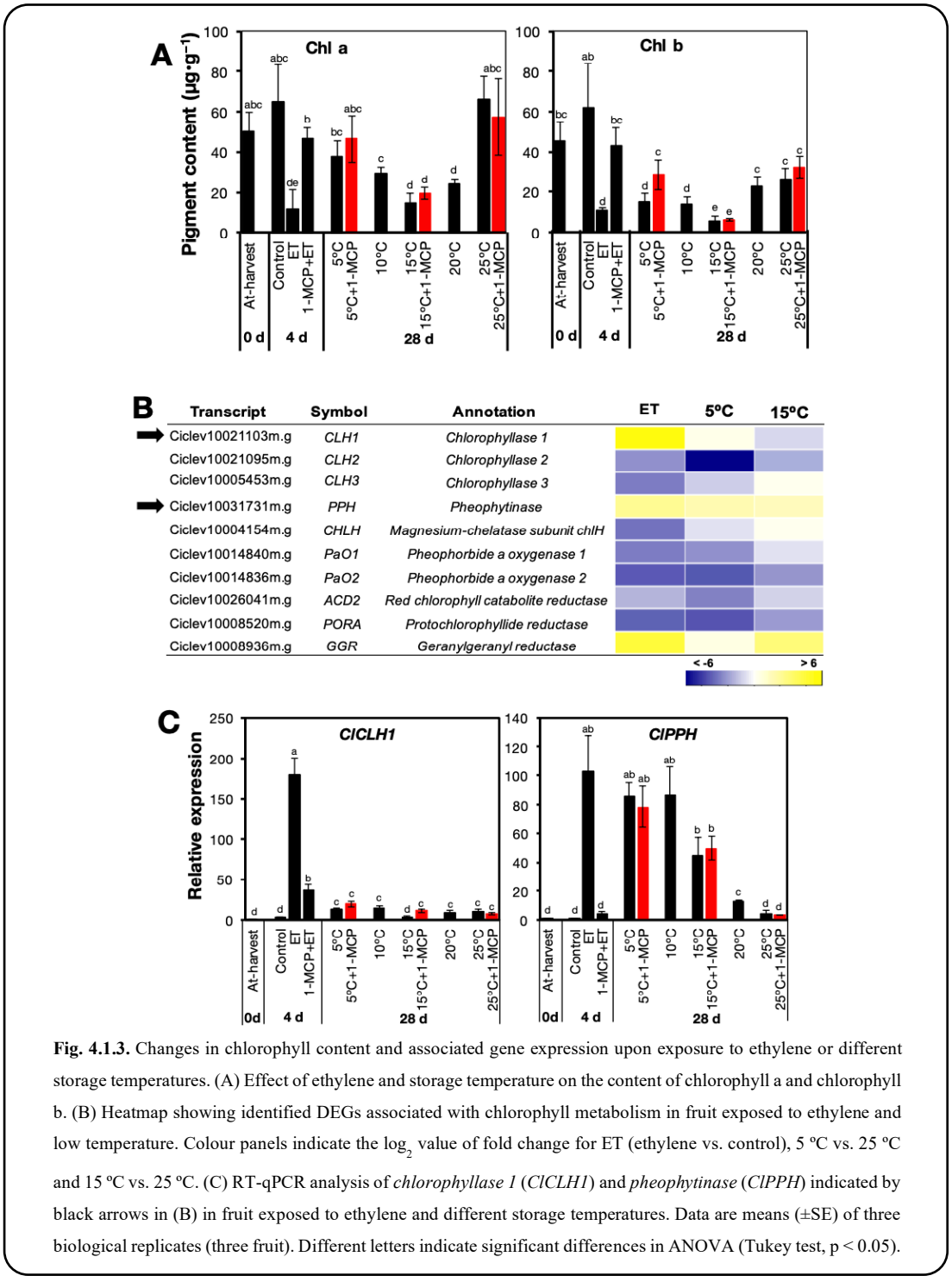


Fig. 4.1.3. Changes in chlorophyll content and associated gene expression upon exposure to ethylene or different storage temperatures. (A) Effect of ethylene and storage temperature on the content of chlorophyll a and chlorophyll b. (B) Heatmap showing identified DEGs associated with chlorophyll metabolism in fruit exposed to ethylene and low temperature. Colour panels indicate the $\log_2$ value of fold change for ET (ethylene vs. control), 5 ºC vs. 25 ºC and 15 ºC vs. 25 ºC. (C) RT-qPCR analysis of *chlorophyllase 1* (*ClCLH1*) and *pheophytinase* (*ClPPH*) indicated by black arrows in (B) in fruit exposed to ethylene and different storage temperatures. Data are means (±SE) of three biological replicates (three fruit). Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

The acceleration of chlorophyll loss by ethylene and low temperature was further verified by examining the expression of chlorophyll metabolism genes. Whereas most of the identified DEGs encoding chlorophyll metabolism enzymes were downregulated, we found three that were upregulated (Fig. 4.1.3B). Among the upregulated genes were *ClCLH1* and *ClPPH*, that had been previously associated with chlorophyll degradation in citrus fruit (Jacob-Wilk *et al*., 1999; Yin *et al*., 2016). Interestingly, *ClCLH1* was up-regulated only by ethylene treatment (which was suppressed by 1-MCP treatment), while *ClPPH* was upregulated by both ethylene treatment and low temperature (Fig. 4.1.3C). It is however worth noting that repeated 1-MCP treatments did not suppress the increased expression of *ClPPH* at 5 ºC and 15 ºC.

Carotenoid metabolism and associated transcripts

Citrus peel degreening is also complemented by a change in the content and composition of carotenoids having varied colours (Kato, 2012; Ohmiya *et al*., 2019). Therefore, we sought to determine the changes in peel carotenoid content triggered by ethylene treatment and storage temperature. Lutein, β-carotene and α-carotene were identified as the major carotenoids in the peel of lemon fruit (Fig. 4.1.4), which was in close agreement with the findings of Agócs *et al*. (2007). Interestingly, the peel content of all the identified carotenoids showed a substantial decrease upon ethylene treatment for 4 d and storage at moderately low temperatures for 28 d (Fig. 4.1.5A). However, while 1-MCP treatment significantly inhibited carotenoid changes induced by ethylene treatment, repeated 1-MCP treatments did not abolish peel carotenoid decrease at 5 ºC and 15 ºC.

By examining the RNA-seq data, we identified 13 DEGs that had been associated with carotenoid metabolism (Fig. 4.1.5B). Out of these, three genes including *ClPSY1*, *ClLCYb2a* and *ClCHYb1* that showed high RPKM values and unique expression patterns were selected for further analysis by RT-qPCR. This analysis revealed that *ClPSY1* and *ClLCYb2a* were upregulated by both ethylene treatment and low temperature, while *ClCHYb1* was upregulated exclusively by low temperature (Fig. 4.1.5C). Additionally, the expression of all the three analysed genes increased in the peel of fruit at 5 ºC and 15 ºC despite the repeated 1-MCP treatments.

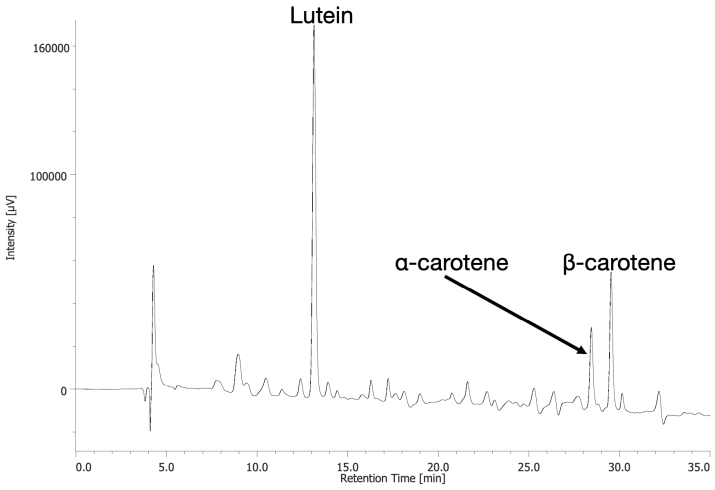


Fig. 4.1.4. Chromatogram showing the major carotenoid pigments identified in the flavedo of lemon fruit.

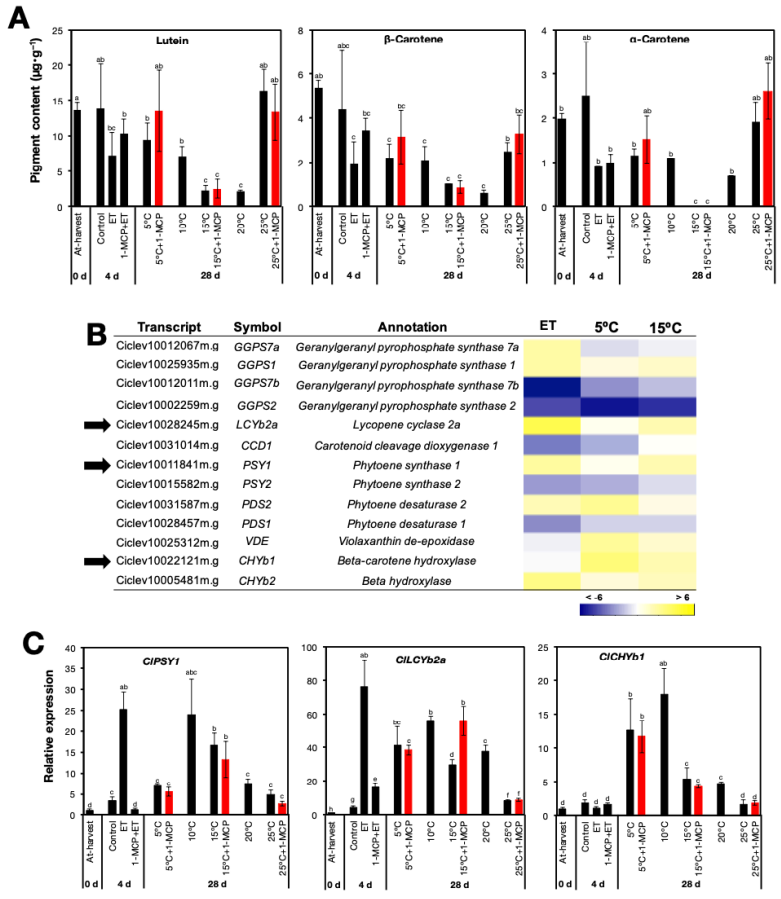


Fig. 4.1.5. Changes in the content of carotenoids and expression of associated metabolism genes upon exposure to ethylene and different storage temperatures. (A) Effect of ethylene and storage temperature on the content of lutein, β-carotene and α-carotene. (B) Heatmap of identified DEGs associated with carotenoid metabolism in fruit exposed to ethylene and low temperature. Colour panels indicate the $\log_2$ value of fold change for ET (ethylene vs. control), 5 ºC vs. 25 ºC and 15 ºC vs. 25 ºC. (C) RT-qPCR analysis of *phytoene synthase 1* (*ClPSY1*), *lycopene cyclase 2a* (*ClLCYb2a*) and *β-carotene hydroxylase 1* (*ClCHYb1*) selected from (B) in fruit exposed to ethylene and different storage temperatures. Data are means (±SE) of three biological replicates (three fruit). Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

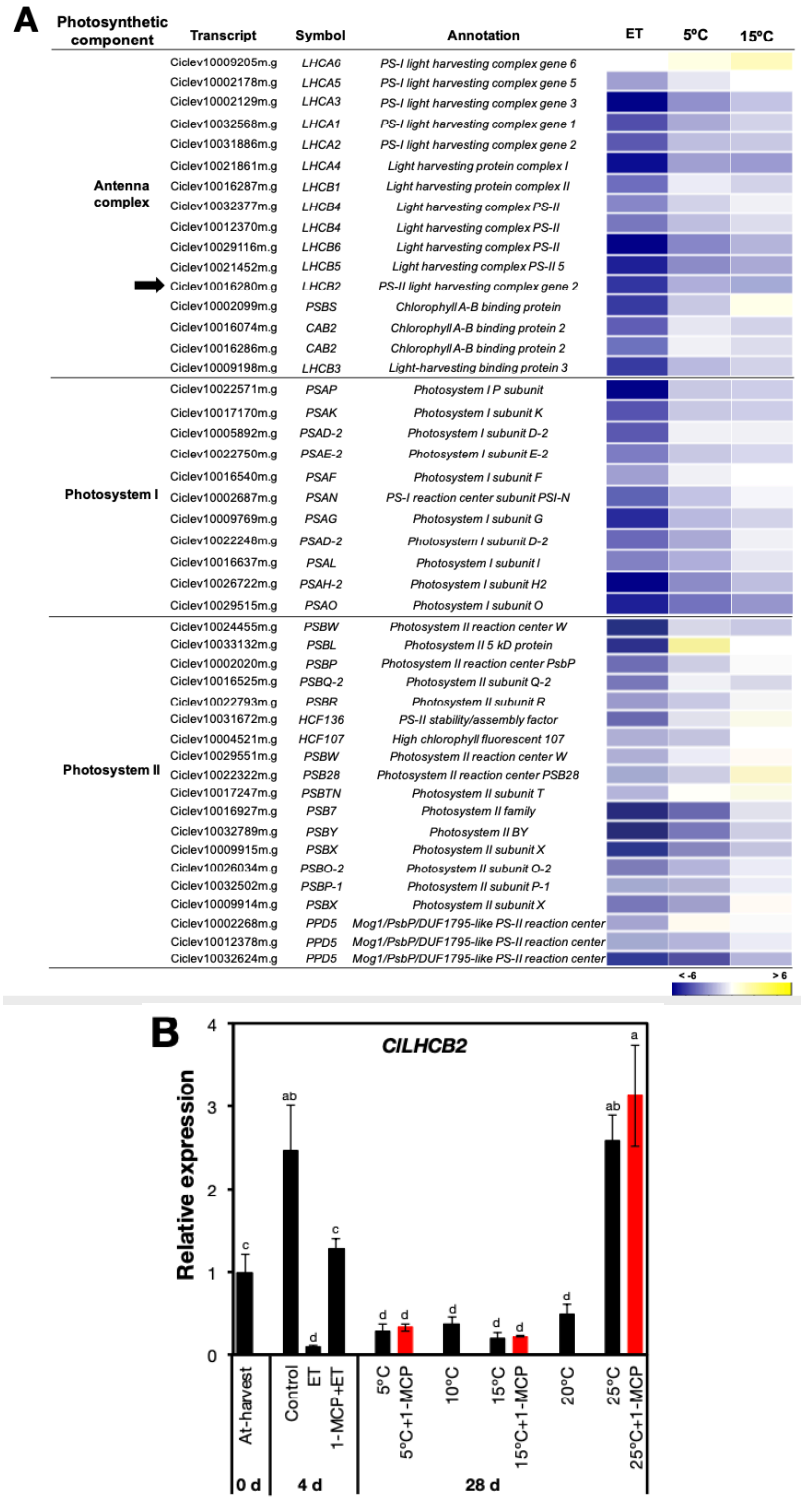


Fig. 4.1.6. Changes in the expression of genes encoding photosystem proteins in response to ethylene and different storage temperatures. (A) Heatmap of identified DEGs encoding photosystem proteins in fruit exposed to ethylene and low temperature. Colour panels indicate the $\log_2$ value of fold change for ET (ethylene vs. control), 5 ºC vs. 25 ºC and 15 ºC vs. 25 ºC. (B) RT-qPCR analysis of *light harvesting complex 2* (*ClLHCB2*) indicated by a black arrow in (A) in fruit exposed to ethylene and different storage temperatures. Data are means (±SE) of three biological replicates (three fruit). Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

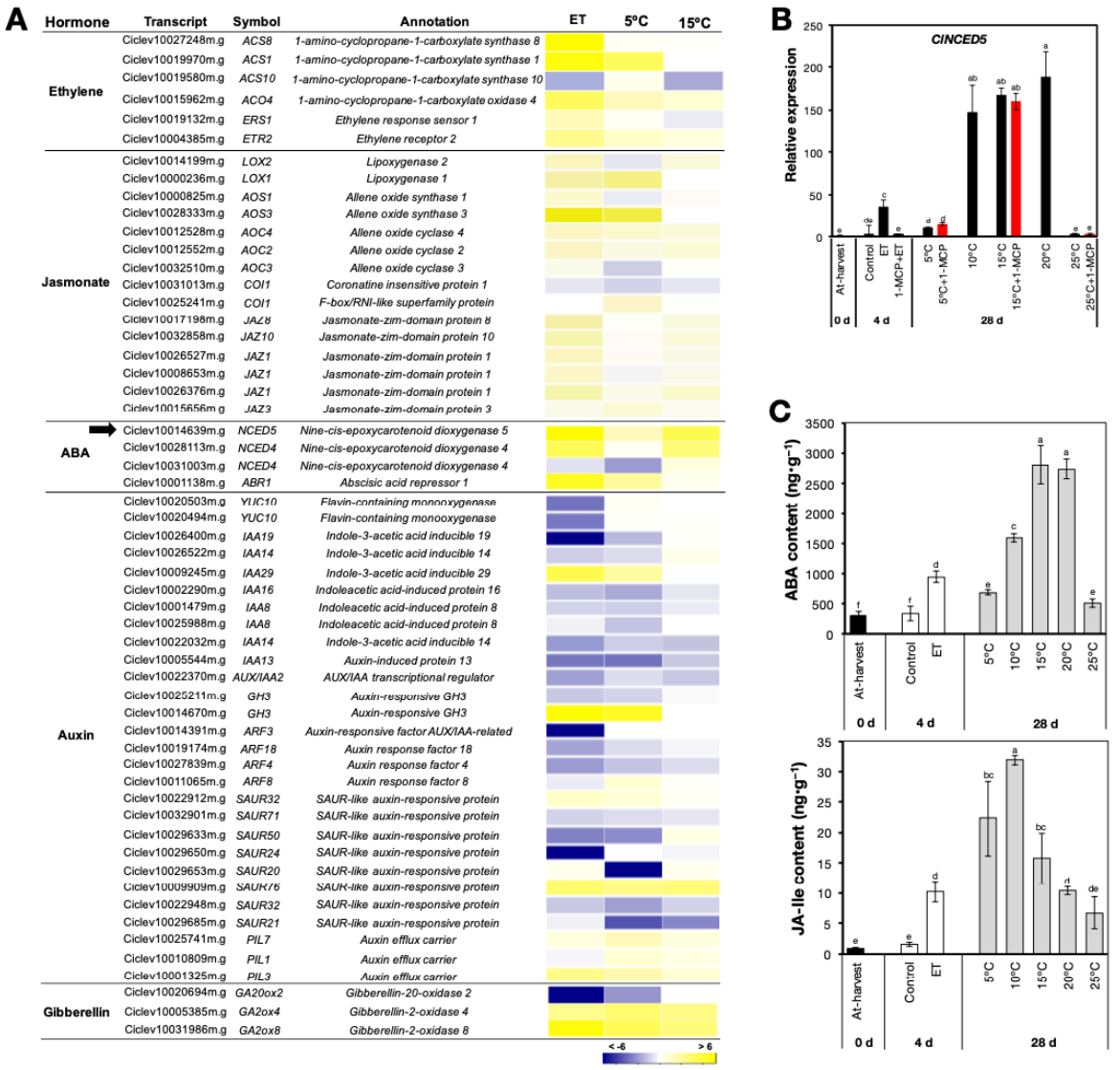


Fig. 4.1.7. Levels of phytohormones and the expression of associated genes in the flavedo of detached lemon fruit. (A) Heatmap showing DEGs encoding proteins associated with phytohormone biosynthesis and signalling in fruit exposed to ethylene and low temperature. Colour panels indicate the $\log_2$ value of fold change for ET (ethylene vs. control), 5 ºC vs. 25 ºC and 15 ºC vs. 25 ºC. (B) RT-qPCR analysis of the ABA biosynthetic gene, *9-cis-epoxycarotenoid dioxygenase 5* (*ClNCED5*), indicated by a black arrow in (A) in fruit exposed to ethylene and different storage temperatures. (C) Levels of ABA and JA-Ile in lemon fruit treated with ethylene and after storage at specified temperatures. Data are means (±SE) of three biological replicates (three fruit). Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

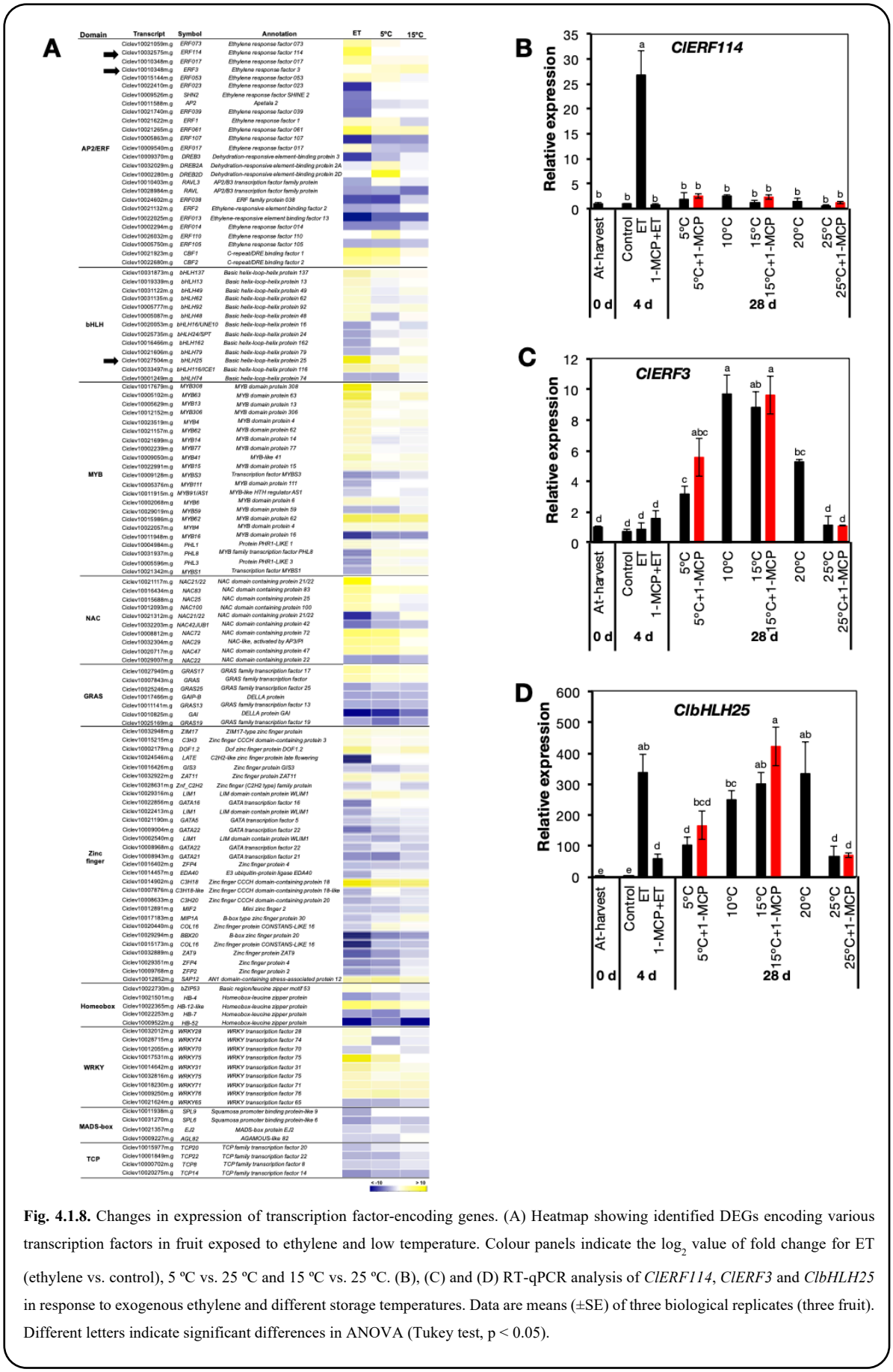


Fig. 4.1.8. Changes in expression of transcription factor-encoding genes. (A) Heatmap showing identified DEGs encoding various transcription factors in fruit exposed to ethylene and low temperature. Colour panels indicate the $\log_2$ value of fold change for ET (ethylene vs. control), 5 ºC vs. 25 ºC and 15 ºC vs. 25 ºC. (B), (C) and (D) RT-qPCR analysis of *ClERF114*, *ClERF3* and *ClbHLH25* in response to exogenous ethylene and different storage temperatures. Data are means (±SE) of three biological replicates (three fruit). Different letters indicate significant differences in ANOVA (Tukey test, $p < 0.05$).

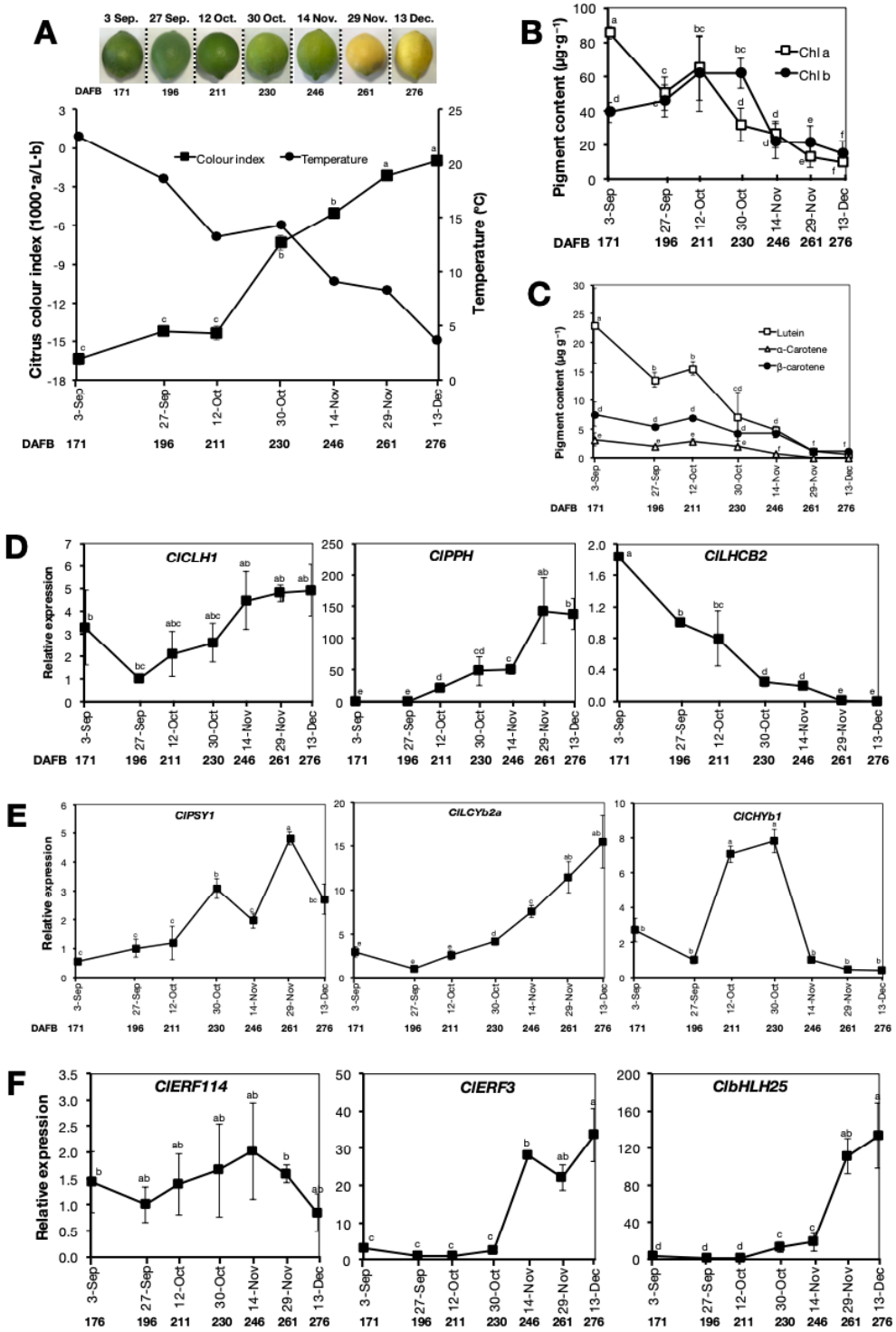


Fig. 4.1.9. Peel colour changes and gene expression analysis in lemon fruit during on-tree maturation. (A) Appearance and citrus colour index of representative fruit at different developmental stages alongside changes in minimum environmental temperatures. Data for minimum temperature were accessed from the website of Japan Meteorological Agency (http://www.data.jma.go.jp/obd/stats/etrn/view/daily_s1.php?prec_no=72&block_no=47891&year=2014&month=12&day=&view=p1). (B) Chlorophyll a and chlorophyll b contents at different developmental stages. (C) Levels of lutein, α-carotene and β-carotene at different developmental stages. RT-qPCR analysis of selected genes associated with chlorophyll metabolism and photosystem proteins (D), carotenoid metabolism (E), and transcription factors (F) at different developmental stages. Data points represent the mean (±SE) of five fruit and different letters indicate significant differences in ANOVA (Tukey's test, $p < 0.05$).

Transcripts encoding photosystem proteins

Genes encoding photosystem proteins featured prominently among the identified DEGs, and most of them were downregulated by both ethylene treatment and low temperature (Fig. 4.1.6A). However, ethylene treatment appeared to have a greater influence on their downregulation than low temperature did. Since most of genes in this category showed a similar expression pattern, we selected only one, *light harvesting complex 2* (*ClLHCB2*) for validation and further analysis by RT-qPCR. Results confirmed that both ethylene treatment and low temperature caused a reduction in the expression of *ClLHCB2* (Fig. 4.1.6B). Nevertheless, repeated 1-MCP treatments did not suppress the expression decrease induced at 5 ºC and 15 ºC, suggesting that the influence of low temperature on *ClLHCB2* expression was independent of ethylene.

Phytohormone levels and associated transcripts

Another prominent category among the identified DEGs included genes that were associated with the biosynthesis and signalling of phytohormones, especially ethylene, JA, ABA, auxin and GA (Fig. 4.1.7A). Most of the ethylene-related genes were up-regulated by ethylene treatment, while low temperature, especially 5 ºC, only showed a slight effect on their expression. On the other hand, genes that were associated with JA and ABA were mostly upregulated by both ethylene treatment and low temperature. Auxin-related genes showed varied expression patterns, although the general trend was towards a downregulation by both ethylene treatment and low temperature. We also identified three GA-associated DEGs of which one (*ClGA20ox2*), which is associated with GA biosynthesis, was downregulated by both ethylene treatment and low temperature, especially at 5 ºC. In contrast, *ClGA2ox4* and *ClGA2ox8* that are associated with GA degradation were upregulated by ethylene treatment as well as low temperature. To verify the roles of ethylene and low temperature in the regulation of phytohormone-related genes, *9-cis-epoxycarotenoid dioxygenase* (*ClNCED5*) which is associated with ABA biosynthesis was chosen for further analysis by RT-qPCR. Results confirmed that *ClNCED5* was upregulated both after 4 d of ethylene exposure, and 28 d of storage at lower temperatures (5 ºC, 10 ºC, 15 ºC and 20 ºC) than 25 ºC (Fig. 4.1.7B). There was also a significant increase in *ClNCED5* expression in fruit that were repeatedly treated with 1-MCP at 5 ºC and 15 ºC. The transcript levels of *ClNCED5* were notably higher in low temperature-stored fruit than in ethylene-treated fruit.

The above changes in expression of phytohormone-associated genes motivated us to determine the phytohormone content in the flavedo of lemon fruit exposed to ethylene and different storage temperatures. The results indicated that both ethylene treatment and low storage temperature caused a significant hike in ABA and JA-Ile levels (Fig. 4.1.7C). In particular, both ABA and JA-Ile levels were substantially higher in fruit stored at low temperatures than in ethylene-treated fruit. Unfortunately, we could not detect the other hormones because of their extremely low endogenous levels and severe ion suppression effects during LC/MS analysis.

Transcripts encoding transcription factors

A total of 128 DEGs in the RNA-seq data were found to encode a wide range of putative TF families including AP2/ERF, bHLH, MYB, NAC, GRAS, zinc finger, homeobox, WRKY, MADS and TCP (Fig. 4.1.8A). This finding underscored the relevance of TF activity in the peel degreening process of lemon fruit. Identified genes were therefore pooled into three distinct groups, which included those that were influenced by (i) ethylene only such as *ClERF114*, (ii) low temperature only such as *ClERF3*, and (iii) both ethylene and low temperature such as *ClbHLH25*. RT-qPCR analysis confirmed that *ClERF114* was exclusively upregulated by ethylene treatment as its expression was maintained at minimal levels during storage (Fig. 4.1.8B). In contrast, *ClERF3* was exclusively upregulated by low temperature since marginal expression levels were registered in ethylene-treated fruit (Fig. 4.1.8C). Finally, *ClbHLH25* expression increased both upon ethylene treatment and after storage at lower temperatures than 25 ºC (Fig. 4.1.8C). It is also noteworthy that repeated 1-MCP treatments failed to abolish the upregulation of *ClERF3* and *ClbHLH25* at 5 ºC and 15 ºC (Fig. 4.1.8B, C).

4.1.3.4. On-tree peel degreening behaviour and expression analysis of associated genes

The roles of ethylene and low temperature in natural peel degreening were further investigated during on-tree maturation of lemon fruit. For this purpose, fruit were harvested at seven progressive stages ranging from 176 to 276 DAFB that occurred between early-September and mid-December. As shown in Fig. 4.1.9A, peel colour progressively changed from green on 3rd September to full yellow on 13th December, which was indicated by a concomitant increase in CCI from -16.3 to -1.1 within the same time span. As peel degreening progressed, the average minimum temperatures in the orchard location decreased gradually from 22.5 ºC on 3rd September to 3.7 ºC on 13th December. The increase in CCI was initially slow between 3rd

September to 12th October from -16.3 to -14.2 when the minimum temperatures were above 13 ºC. Interestingly, CCI increased rapidly from -14.2 to -1.1 between 12th October and 13th December when the minimum temperatures were maintained at below 13 ºC. The observed loss of green colour during on-tree maturation was in close agreement with a gradual decrease in the peel chlorophyll a and b contents (Fig. 4.1.9B). Equally, peel degreening was also accompanied by a gradual decline in the peel content of lutein, β-carotene and α-carotene (Fig. 4.1.9C).

The correlation between peel colour changes and environmental temperature drops was further investigated by examining the expression patterns of selected genes induced by ethylene and/or low temperature from the RNA-seq data. On-tree peel degreening coincided with an upregulation of *ClPPH* and downregulation of *ClLHCB2* (Fig. 4.1.9D), both of which were earlier shown to be influenced by low temperature (Fig. 4.1.3C, 4.1.6B). However, the ethylene-specific *ClCLH1* did not show any significant changes in expression. On-tree peel degreening was also accompanied by the upregulation of all the three analysed carotenoid metabolism genes *ClPSY1*, *ClLCYb2a* and *ClCHYb1* (Fig. 4.1.9E), which were earlier shown to be upregulated by low temperature (Fig. 4.1.5C). Among the TF-encoding genes, the ethylene-specific *ClERF114* did not show any significant expression changes, whereas both *ClERF3* and *ClbHLH25* were upregulated especially from 30th October when the minimum temperatures were below 13 ºC (Fig. 4.1.9F). Altogether, these observations demonstrated strong similarities between on-tree and low temperature-modulated peel degreening, as well as their dissimilarities with ethylene-induced changes.

4.1.4. Discussion

Many studies have shown that ethylene regulates peel degreening in citrus fruit (Purvis and Barmore, 1981; Shemer *et al*., 2008; Yin *et al*., 2016), prompting its wide use for commercial degreening purposes (Porat, 2008; Mayuoni *et al*., 2011). This is consistent with the present study as ethylene treatment induced rapid peel degreening in detached lemon fruit (Fig. 4.1.1A). However, the important regulators involved in natural peel degreening remain a mystery since citrus fruit are considered non-climacteric, and thus produce trace levels of endogenous ethylene (system I ethylene) (Katz *et al*., 2004). Previous studies have demonstrated that there is a close association between low temperature and peel colouration in multiple citrus fruit

species (Carmona *et al*., 2012a; Manera *et al*., 2012; Manera *et al*., 2013), but the molecular mechanisms involved are unclear. In the present work, we present conclusive data demonstrating that low temperature can transcriptionally modulate natural peel degreening in lemon fruit independently of the ethylene signal.

Results obtained in this study demonstrate very clearly that moderately low storage temperatures promoted peel degreening (Fig. 4.1.1B). Because of the known involvement of ethylene in citrus degreening (Fig. 4.1.1A), peel colour changes that occur during low temperature storage have often been attributed to ethylene signalling, that is, trace levels of physiologically active system I ethylene are thought to be bound in tissues (Goldschmidt *et al*., 1993; Carmona *et al*., 2012b). Ethylene-induced degreening is completely inhibited by pre-treatment with 1-MCP (Fig. 4.1.1A; Jomori *et al*., 2003; McCollum and Maul, 2007; Li *et al*., 2016). 1-MCP treatment also inhibits the ripening process in fruit that have a strong requirement for ethylene to ripen (Watkins, 2006). In higher plants, ethylene receptors act as negative regulators (Hua and Meyerowitz, 1998), and their binding by ethylene subjects them to degradation via the ubiquitin-proteasome pathway (Kevany *et al*., 2007). 1-MCP is assumed to irreversibly bind and phosphorylate ethylene receptors (Kamiyoshihara *et al*., 2012), with a higher affinity than ethylene (Jiang *et al*., 1999), resulting in relatively stable complexes that suppress ethylene signalling even in the presence of ethylene. If endogenous ethylene was physiologically active, then its action should be suppressed by the application of ethylene antagonists such as 1-MCP. However, it is surprising that peel degreening elicited by low temperature was not abolished by repeated 1-MCP treatments (Fig. 4.1.1C), which indicated that it most likely occurred in an ethylene-independent manner.

Further evidence for this conclusion is the identification of distinct gene sets that are regulated by either ethylene or low temperature in the flavedo of lemon fruit (Fig. 4.1.2). Ethylene-specific genes such as *ClCLH1* and *ClERF114* were not differentially expressed during low temperature storage (Fig. 4.1.3C, 4.1.8B), which implies that ethylene signalling was non-functional in stored fruit. Additionally, ethylene treatment did not show any significant effect on the expression of another distinct gene set that were influenced by low temperature, including *ClCHYb1* and *ClERF3* (Fig. 4.1.5C, 4.1.8C). This is perhaps the most direct evidence for an ethylene-independent modulation of peel degreening by low temperature. Although the third gene set, including *ClPPH*, *ClPSY1*, *ClLCYb2a*, *ClLHCB2*, *ClNCED5* and *ClbHLH25* were differentially regulated by either ethylene or low temperature (Fig. 4.1.3C; 4.1.5B, C;

4.1.6B; 4.1.7B; 4.1.8D), their stimulation by low temperature was not altered by repeated 1-MCP treatments, excluding any likelihood of ethylene involvement during storage.

The degreening observed in lemon fruit exposed to ethylene is, in all likelihood, due to a reduction in peel chlorophyll content (Fig. 4.1.3A), which could be attributed to the upregulation of *ClCLH1* and *ClPPH* (Fig. 4.1.3C). Ethylene-induced peel chlorophyll degradation in citrus fruit has also been linked to increased transcript levels of homologues of *ClCLH1* (Jacob-Wilk *et al*., 1999; Shemer *et al*., 2008; Yin *et al*., 2016), and *ClPPH* (Yin *et al*., 2016). During storage, however, the minimal expression levels of *ClCLH1* excluding any possibility that it might be involved in low temperature-triggered chlorophyll degradation. Instead, the degradation of chlorophylls caused by low temperature can be attributed to the ethylene-independent upregulation of *ClPPH*, which is known to encode an enzyme with a similar dephytilation activity as CLH (Schelbert *et al*., 2009).

Peel carotenoid content decreased upon degreening in response to both ethylene and low temperature (Fig. 4.1.5A). This decrease is not uncommon as previous studies have also demonstrated that the peel content of carotenoids, especially lutein, in lemon fruit decreased during maturation (Kato, 2012; Conesa *et al*., 2019). Nevertheless, the yellowish appearance of degreened lemon fruit (Fig. 4.1.1) could be attributed to the small but significant levels of lutein, β-carotene and α-carotene (Fig. 4.1.5A), which might be intensified by their unmasking brought about by the loss of chlorophyll. Changes in peel carotenoid content are initiated by the expression of various carotenoid metabolism-related genes which can be stimulated by either ethylene or low temperature (Fig. 4.1.5B; Matsumoto *et al*., 2009; Rodrigo and Zacarias, 2007). In this study, however, it appears that carotenoid metabolism-associated genes such as *ClPSY1*, *ClLCYb2a* and *ClCHYb1* are also transcriptionally modulated by low temperature independently of ethylene.

The degradation of chlorophylls caused by exposure to either ethylene or low temperature could also be facilitated by changes in photosystem proteins. The disruption of pigment-protein complexes is thought to be a crucial step in the chlorophyll degradation pathway (Barry, 2009). Consequently, the stay-green protein (SGR), which encodes a Mg-dechelatase (Shimoda *et al*., 2016), has been shown to aid the dis-aggregation of photosystem proteins, particularly the light-harvesting chlorophyll a/b binding (CAB) complex (Jiang *et al*., 2011; Sakuraba *et al*., 2012). Because photosystem proteins bind pigments, a large drop in their transcripts caused by

ethylene or low temperature (Fig. 4.1.6) would possibly favour the accumulation of free chlorophylls that can easily be accessed by degradatory enzymes. Peng *et al*. (2013) also reported that the transcript levels of *CitCAB1* and *CitCAB2* drastically decreased during ethylene-induced and natural peel degreening in 'Ponkan' mandarins. However, the results of the present study suggest that the decrease in the expression of photosystem-encoding genes during natural peel degreening could be stimulated by low temperature independently of ethylene.

Besides ethylene, various phytohormones such as ABA, GA and JA have been implicated in the peel colour changes that occur during citrus fruit maturation. Peel degreening was shown to be accompanied by an increase in ABA content (Goldschmidt *et al*., 1973), as well as in the expression of ABA biosynthetic and signalling elements (Rodrigo *et al*., 2006; Kato *et al*., 2006). In addition, exogenous ABA accelerated fruit ripening and enhanced fruit colour development (Wang *et al*., 2016), while ABA-deficient citrus mutants showed a delay in the rate of peel degreening (Rodrigo *et al*., 2003). In this study, ABA levels increased in ethylene-treated and low temperature-stored fruit (Fig. 4.1.7C), accompanied by an increase in the expression of ABA biosynthetic and signalling genes (Fig. 4.1.7A, B). These findings, together with previous reports, suggest that ABA has a positive regulatory role in either ethylene-induced or low temperature-modulated peel degreening in lemon fruit. GA, on the other hand, is known to retard peel colour change. GA application on green citrus fruit was shown to cause a significant delay in peel colour break (Alós *et al*., 2006; Rodrigo and Zacarias, 2007; Rios *et al*., 2010). It is therefore logical that the transcript levels of the GA biosynthetic gene (*ClGA20ox2*) would decrease, whereas those of GA degradatory genes (*ClGA2ox4* and *GA2ox8*) would increase during peel degreening caused by either ethylene or low temperature (Fig. 6A). JAs have thus far been studied in the context of plant adaptive responses to various biotic and abiotic stresses (Zhang *et al*., 2019). However, JA has also been shown to promote fruit ripening in citrus (Zhang *et al*., 2014), strawberry (Concha *et al*., 2013) and tomato (Liu *et al*., 2012). This is consistent with the present findings as lemon fruit degreening was accompanied by an increase in the levels of JA-Ile (Fig. 4.1.7C), which is the active conjugate of JA. Additionally, the expression of a large number of JA biosynthetic and signalling-related genes were independently upregulated by either ethylene treatment or low temperature (Fig. 4.1.7A).

Developmentally regulated plant processes such as peel degreening are typically influenced by TFs. Various TFs such as AtNAC046, AtPIF4, AtPIF5, AtORE1 and AtEIN3 were shown to significantly enhance leaf senescence in Arabidopsis by promoting the activity of chlorophyll degradation-related genes (Song *et al*., 2014; Qiu *et al*., 2015; Zhang *et al*., 2015; Oda-Yamamizo *et al*., 2016). In broccoli, MYB, bHLH, and bZIP gene families were associated with chlorophyll metabolism while NACs and ERFs regulated carotenoid biosynthesis (Luo *et al*., 2019). CitERF6 and CitERF13 have also been associated with chlorophyll degradation during ethylene-induced and natural peel degreening in citrus (Yin *et al*., 2016; Li *et al*., 2019). In the present work, the expression patterns of genes encoding a wide range of TF families suggested that ethylene-induced and low temperature-modulated peel degreening pathways were distinct in lemon fruit (Fig. 4.1.8). Therefore, ethylene-induced degreening is most likely to be regulated by ethylene-specific TFs such as *ClERF114* (Fig. 4.1.8B) and shared TFs such as *ClbHLH25* (Fig. 4.1.8D). In contrast, low temperature-modulated degreening could be regulated by specific TFs such as *ClERF3* (Fig. 4.1.8C), as well as shared ones such as *ClbHLH25* (Fig. 4.1.8D).

In this study, low temperature appears to also play a prominent role in natural peel degreening during on-tree lemon fruit maturation. Peel degreening and the associated reduction in the content of chlorophylls and carotenoids coincided with gradual drops in minimum environmental temperatures, to below 13 ºC (Fig. 4.1.9A–C). Similar to our study, previous studies have also demonstrated that peel degreening in most citrus fruit progresses as the environmental temperature decreases (Manera *et al*., 2012; Manera *et al*., 2013; Rodrigo *et al*., 2013; Conesa *et al*., 2019). It is intriguing that *ClCLH1* and *ClERF114*, which were earlier shown to exhibit an ethylene-specific pattern (Fig. 4.1.3C, 4.1.8B) did not show any significant changes in expression during on-tree peel degreening (Fig. 4.1.9D, F). Earlier studies have also demonstrated that *ClCLH1* homologues in other citrus fruit exhibit a dramatic induction in response to ethylene treatment, yet lack a measurable expression increase during natural degreening (Jacob-Wilk *et al*., 1999; Yin *et al*., 2016). Here, we suggest that this discrepancy could be due to a lack of a functional ethylene signalling during on-tree maturation, given that *ClCLH1* is only upregulated in the presence of ethylene (Fig. 4.1.3C). In contrast, genes that responded to low temperature during storage such as *ClPPH*, *ClLHCB2*, *ClPSY1*, *ClLCYb2a*, *ClCHYb1*, *ClERF3*, *ClbHLH25* also exhibited similar expression patterns during on-tree maturation (Fig. 4.1.9D–F), indicating that they were involved in the on-tree peel degreening processes. These similarities between low temperature-induced and on-tree gene expression

patterns, coupled with their dissimilarities to ethylene-induced changes, provide clear evidence to suggest that on-tree peel degreening responses are modulated by low temperature independently of ethylene in lemon fruit.

It is also important to note that many genes were differentially expressed in fruit at 5 ºC, yet the peel degreening rate was significantly slower than in fruit at 10 ºC, 15 ºC and 20 ºC. This is probably due to low activity of peel degreening-associated enzymes at 5 ºC, as low temperature is known to decrease enzyme activity in plants (Jin *et al*., 2009; Yun *et al*., 2012).

The regulation of fruit ripening by low temperature is not unique to citrus fruit. Previous studies have also demonstrated a role for low temperature, either independently or in concert with ethylene, in the regulation of fruit ripening in multiple fruit species such as kiwifruit (Mworia *et al*., 2012; Asiche *et al*., 2017; Asiche *et al*., 2018; Mitalo et al., 2018a; Mitalo *et al*., 2019a; Mitalo *et al*., 2019b), pears (El-Sharkawy et al., 2003; Mitalo *et al*., 2019c) and apples (Tacken *et al*., 2010). From an ecological perspective, the primary purpose of fruit ripening is to make fruit attractive to seed-dispersing organisms. To ensure their future survival, most temperate fruit are faced with the challenge of dispersing their seeds in time before the onset of harsh winter conditions. Therefore, environmental temperature drops associated with autumn might provide an alternative stimulus for fruit ripening induction in fruits that lack a functional ethylene signalling pathway during maturation, like citrus.

4.1.5. Conclusion

In conclusion, the present work provides a comprehensive overview of ethylene- and low temperature-induced peel degreening responses during maturation in lemon fruit by comparing physiochemical changes and corresponding transcriptome changes. Both ethylene and low temperature promote peel degreening by inducing transcriptome changes associated with chlorophyll degradation, carotenoid metabolism, photosystem disassembly, phytohormones and TFs. However, blocking ethylene signalling by repeated 1-MCP treatments does not eliminate low temperature-induced changes. On-tree peel degreening, which typical occurs as minimum environmental temperature drops, corresponds with the differential regulation of low temperature-regulated genes whereas genes that uniquely respond to ethylene do not exhibit any significant expression changes. These data suggest that low temperature plays a prominent

role in promoting natural peel degreening both on and off the tree. In our further studies, we aim to identify the direct and indirect targets of low temperature-regulated transcripts that have been uncovered in this study towards elaboration of the molecular bases for low temperature modulation of peel degreening and fruit ripening in general.

4.2. Physiological and transcriptomic evidence suggests an ethylene-independent regulation of satsuma mandarin fruit degreening by low temperature

Abstract

Peel degreening in response to propylene treatment and different storage temperatures was characterized in satsuma mandarin (*Citrus unshiu* Marc. cv. Aoshima) fruit. Continuous propylene treatment triggered a rapid increase in citrus colour index (CCI) and reduction in chlorophyll content within 4–6 d. During storage, peel CCI increased and chlorophyll content decreased in fruit at 10 ºC and 15 ºC after 28–42 d while there were no substantial changes at 5 ºC, 20 ºC and 25 ºC. RNA-seq analysis unveiled distinct genes that were differentially regulated by ethylene and low temperature. In particular, ethylene exclusively regulated a particular set of genes including *CuCOPT1* and *CuPOX-A2* while low temperature uniquely regulated a distinct set that included genes such as *CuERF3*. Initiation of on-tree peel degreening coincided with the start of autumnal drops in environmental temperature, and this was accompanied by differential expression of low temperature-regulated genes. Contrarily, genes exclusively regulated by ethylene did not show any significant change in expression during on-tree peel degreening. These findings suggest that low temperature-triggered peel degreening in satsuma mandarins is independent of ethylene signalling.

4.2.1. Introduction

Fruit ripening is a complex process involving various physiological and biochemical modifications in colour, texture, flavour and aroma profiles (Seymour *et al*., 2014), which are widely known to be under hormonal, nutritional and environmental control (Giovannoni, 2004). In citrus fruit, such as satsuma mandarins (*Citrus unshiu* Marc.), the most conspicuous change during fruit ripening is peel degreening resulting from degradation of chlorophylls and metabolism of carotenoids (Iglesias *et al*., 2007). Peel colour is one of the main determinants of citrus fruit quality and marketability (Porat, 2008).

The plant hormone ethylene has profound effects on fruit ripening. Fruit have thus been classified into two major groups, climacteric and non-climacteric, based on their ripening responses to ethylene (Giovannoni, 2004). In climacteric fruit such as tomato, there is a sharp increase in ethylene production (system II) at the onset of ripening which is considered to control the changes in colour, aroma, texture and other attributes (Lelievre *et al*., 1997a; Cara

and Giovannoni, 2008). Fruit ripening is inhibited in mutants or transgenic lines exhibiting reduced ethylene production and perception (Barry *et al*., 2000; Giovannoni, 2007), and by blocking ethylene signalling using chemical antagonists such as 1-methylcyclopropene (1-MCP) (Sisler and Serek, 1997). Additionally, exogenous application of ethylene (or its analogue, propylene) triggers fruit ripening in climacteric fruit (Barry *et al*., 2000; Hiwasa *et al*., 2003; Inaba *et al*., 2007). On the other hand, non-climacteric fruit such as grapes, cherries and strawberries produce trace levels of ethylene (system I) throughout the ripening process, and ethylene treatments are ineffective with regard to ripening (Giovannoni, 2004).

Citrus fruit exhibit a unique non-climacteric ripening behaviour as ethylene treatment can enhance peel degreening by elevating the expression levels of genes associated with chlorophyll degradation (Fujii *et al*., 2007; Yin *et al*., 2016), and carotenoid metabolism (Fujii *et al*., 2007; Rodrigo and Zacarias, 2007). This observation has led to a wide use of the hormone (ethylene) in commercial degreening practices to advance or attain uniform peel colour development (Porat, 2008; Mayuoni *et al*., 2011). However, whether ethylene plays a role in natural peel degreening remains unclear as mature citrus fruit produce trace levels of system I ethylene (Eaks, 1970; Sawamura, 1981; Katz *et al*., 2004).

Low temperature is another factor that affects citrus fruit degreening. It has been shown in multiple citrus species that cold periods below 13 ºC are required for normal peel degreening during natural maturation (Manera *et al*., 2012; Manera *et al*., 2013; Rodrigo *et al*., 2013; Conesa *et al*., 2019). In Japan, the initiation of peel degreening in satsuma mandarins occurs in autumn when the environmental temperatures begin to drop (personal observation). Independent studies have also demonstrated that low/intermediate storage temperatures (6–15 ºC) promote peel degreening in citrus fruit by increasing chlorophyll degradation and carotenoid metabolism rates (Matsumoto *et al*., 2009; Van Wyk *et al*., 2009; Zhu *et al*., 2011; Carmona *et al*., 2012a; Tao *et al*., 2012). However, unlike for ethylene, very little is known about the precise role of low temperature in the degreening process of citrus fruit.

A transcriptome analysis could be one of the most powerful tools to understand the intricate transcriptional regulation during fruit development and ripening. Various studies have employed transcriptome analysis (mostly by RNA-seq) to understand ethylene regulatory mechanisms during fruit ripening in many climacteric fruit including tomatoes (Fujisawa *et al*., 2013), bananas (Asif *et al*., 2014), and pears (Hao *et al*., 2018). In kiwifruit, transcriptome

analyses were used to demonstrate that certain ripening-related genes are regulated by low temperature independently of ethylene (Asiche *et al*., 2018; Mitalo *et al*., 2019a; Mitalo *et al*., 2019b). Transcriptomic studies in European pear fruit also showed that low temperature-specific genes might be involved in the promotion of fruit ripening (Nham *et al*., 2017; Mitalo *et al*., 2019c). Therefore, transcriptomic data, coupled with physiological evidence, will provide new insights into the low temperature regulatory mechanisms involved in citrus fruit degreening.

In the present study, peel degreening changes induced by ethylene and low temperature were examined using RNA-seq in satsuma mandarin fruit. Changes in peel colour index and chlorophyll content were monitored in response to continuous treatment with propylene, a well-known ethylene analogue (McMurchie *et al*., 1972), and during storage at different temperatures including 5 ºC, 10 ºC, 15 ºC, 20 ºC and 25 ºC. Further, RNA-seq analysis followed by confirmatory RT-qPCR were carried out to distinguish ethylene and low temperature-induced transcriptional changes. The overall objective was to determine whether ethylene plays a role in peel degreening changes that are induced by low/intermediate temperature.

4.2.2. Materials and methods

4.2.2.1. Plant material

Satsuma mandarin fruit (*Citrus unshiu* Marc. cv. 'Aoshima') grown under standard cultural practices were obtained from a commercial orchard in Takamatsu (Kagawa, Japan). Fruit were harvested in 2017 at a physiological maturity stage (when endogenous ethylene production levels were minute). After harvesting, careful sorting was carried out to exclude fruit with physical injuries and disease symptoms, and to ensure uniform size before randomly assigning them to treatments.

4.2.2.2. Treatments

To determine postharvest ethylene effect, two sets of 20 fruit each were used. The first set of fruit were placed in gas-tight plastic containers that were continuously supplied with 5000 µL L^{-1} propylene for up to 6 d to trigger ethylene signalling. The second set contained non-treated fruit as a control. Both propylene-treated and control fruit were held at 25 ºC. Soda lime was placed in containers used for propylene treatment to prevent CO_2 accumulation. For

postharvest storage tests, five sets of 70 fruit each were stored either at 5 ºC, 10 ºC, 15 ºC, 20 ºC and 25 ºC for up to 42 d. Ethylene production patterns of each fruit were monitored regularly and any fruit that exhibited increased ethylene production rates (mostly due to disease infection) were removed from the storage chambers. In this way, only fruit that did not show any measurable increase in ethylene production were used for the analyses presented in this study. Sampling for on-tree peel degreening assessment was from seven harvests during fruit development: 6 Sep., 20 Sep., 5 Oct., 21 Oct., 7 Nov., 18 Nov., and 6 Dec. Fruit peel was sampled, frozen in liquid nitrogen and stored at -80 ºC for future analysis. Each sample contained three biological replicates.

4.2.2.3. Determination of colour and chlorophyll content

Colour measurements were carried out with a Minolta CR-200B chromameter (Konica Minolta, Tokyo, Japan) at evenly distributed equatorial sites. The citrus colour index (CCI) values were presented as the results of 1000•a/L•b transformation, expressed as a mean of five fruit.
Chlorophylls were extracted and quantified according to the procedure by Rodrigo et al. (2003) with slight modifications. After extraction in 80 % acetone, appropriated dilutions were used to measure absorbance at 646.8 nm and 663.2 nm. Chlorophyll content was calculated from these measurements using Lichtenthaler and Wellburn equations (Wellburn, 1994).

4.2.2.4. RNA extraction

Total RNA was extracted in triplicate from ~2 g of flavedo tissue according to the procedure by Ikoma et al. (1996) with slight modifications. Treatment with DNase I (Nippon Gene, Tokyo) followed by clean-up using FavorPrep after Tri-Reagent RNA Clean-up Kit (Favorgen Biotech Co., Pingtung, Taiwan) were carried out to remove genomic DNA contamination.

4.2.2.5. RNA-seq and differential gene expression analysis

RNA extracted from the flavedo of control and propylene-treated fruit (after 4 d), as well as fruit stored at 5 ºC, 10 ºC, 15 ºC, 20 ºC and 25 ºC for 28 d was subjected to transcriptome analysis via RNA-seq. Illumina paired-end libraries were constructed using NEBNext® Ultra™ RNA Library Prep Kit for Illumina (New England Biolabs, MA, USA).The libraries were then sequenced on an Illumina HiSeq-2500 platform (Hokkaido System Co. Ltd., Japan). Generated reads were trimmed to obtain ≥ 10 million paired reads per sample before being mapped to the reference *Citrus clementina* Genome v1.0 (Wu *et al*., 2014). Gene expression

levels were calculated using the reads per kilobase per million (RPKM) method. Differentially expressed genes (DEGs) were identified using the false discovery rates (FDR) analysis (Robinson et al., 2010). Genes with RPKM ≥ 3.0, FDR ≤ 0.001, and fold change ≥ 3.0 were considered as DEGs. Non-treated samples were used as a control in the determination of propylene effect. For storage tests, samples obtained at 25 ºC were used as a control. Modules of highly correlated genes were generated using the WGCNA method (Zhang and Horvath, 2005; Langfelder and Horvath, 2008).

4.2.2.6. Reverse-transcriptase quantitative PCR (RT-qPCR) analysis

The method used was similar to that used in our previous studies (Mitalo *et al*., 2019a; Mitalo *et al*., 2019c). Briefly, cDNA was synthesized from 2.4 µg total RNA using Takara RNA PCR kit (Takara, Kyoto, Japan). Gene-specific primers (Appendix 9) were designed using Primer3 software (version 0.4.0, http://bioinfo.ut.ee/primer3-0.4.0/). Gene expression was examined in triplicate on a MYiQ Single-Color Reverse Transcriptase-Quantitative PCR Detection System (Bio-Rad, Hercules, CA, USA) using TB Green™ Premix ExTaq™ II (Takara, Kyoto, Japan). Primer specificity was verified by melting curve analysis. *CuActin* (Ciclev10025866m.g) was used as the housekeeping gene after examining its constitutive expression pattern from the RNA-seq results. Relative expression values were calculated with samples obtained on 6 Sep. calibrated as 1.

4.2.2.7. Statistical analysis

Data presented in this study were subjected to statistical analysis using R (version 3.6.2). ANOVA followed by post-hoc Tukey's test ($P < 0.05$) were used to detect statistical differences in CCI, chlorophyll content and gene expression among the treatments.

4.2.3. Results

4.2.3.1. Effect of propylene treatment on postharvest peel degreening

Satsuma mandarin fruit were treated with propylene (at 25 ºC) to initiate ethylene signalling. Loss of green colour was observed in the fruit peels after 2 d of treatment with propylene, with fruit attaining appreciable yellow colour after 6 d (Fig. 4.2.1A). Peel CCI also increased rapidly from -21.0 at 0 d to -10.5, -4.3 and -0.1 after 2, 4 and 6 d, respectively, of propylene treatment (Fig. 4.2.1B). Concomitant with the above colour changes, propylene treatment resulted in a drastic decrease in peel chlorophyll a content from the initial 300 µg g^{-1} at 0 d to 196 µg g^{-1}

and 53 µg g^{-1} after 2 and 4 d, respectively, and to undetectable levels after 6 d (Fig. 4.2.1C). Equally, peel chlorophyll b content decreased from 135 µg g^{-1} at 0 d to 73 µg g^{-1} after 2 d, 23 µg g^{-1} after 4 d and undetectable levels after 6 d (Fig. 4.2.1C). On the other hand, there were no appreciable changes in peel colour and chlorophyll content in non-treated (control) fruit throughout the experimental duration. These findings confirm that ethylene signalling plays a vital role in the regulation of peel degreening changes in satsuma mandarins despite their non-climacteric ripening nature.

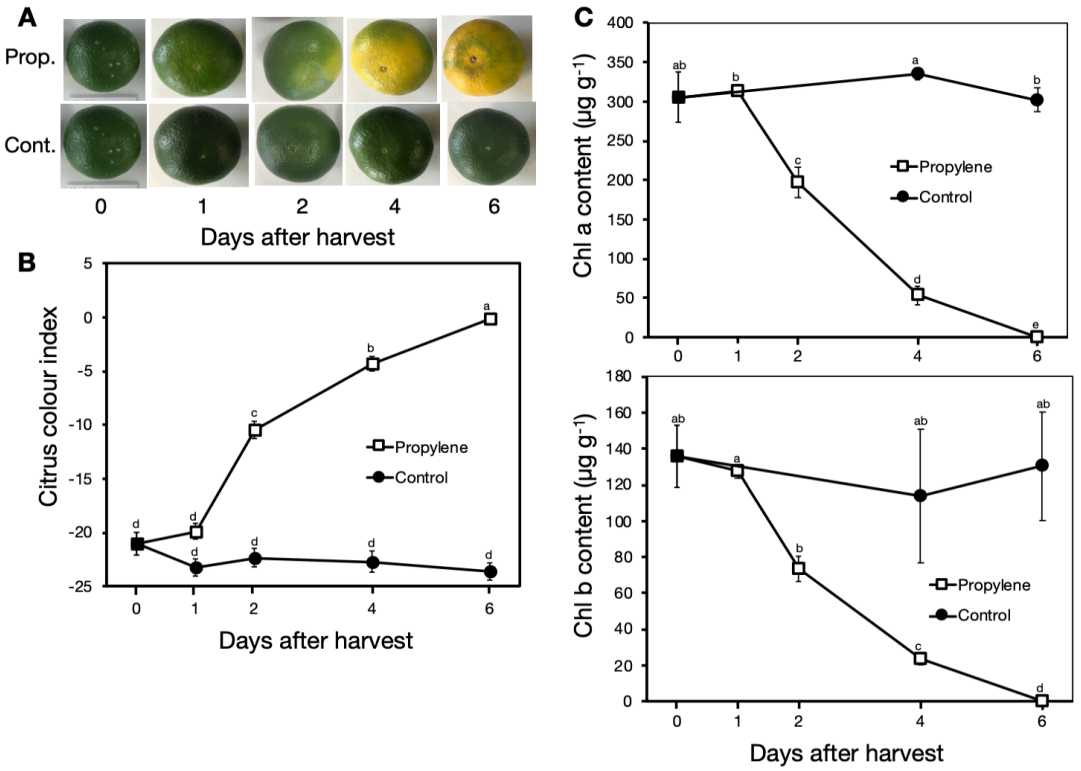


Fig. 4.2.1. Ethylene-induced peel degreening in satsuma mandarin fruit. (A) Appearance of fruit during treatment with propylene, an analogue of ethylene. (B) Changes in citrus colour index and (C) chlorophyll content in response to propylene treatment. Propylene treatment was carried out continuously at 25 ºC. Data points represent the mean (± SE) of five fruit and different letters indicate significant differences in ANOVA (Tukey's test, $p < 0.05$).

4.2.3.2. Peel degreening rates at different storage temperatures

The peel degreening behaviour of satsuma mandarin fruit was also examined during storage at 5 ºC, 10 ºC, 15 ºC, 20 ºC and 25 ºC in ethylene-free chambers. Fruit stored at 10 ºC and 15 ºC started to lose their green peel colour after 14 d with simultaneous acquisition of yellow colour which became intense after 42 d (Fig. 4.2.2A). The peel CCI also increased during storage at 10 ºC and 15 ºC from -21 at 0 d to about -5 and -2 after 28 and 42 d, respectively (Fig. 4.2. 2B). Chlorophyll measurements revealed that storage at 10 ºC and 15 ºC resulted in a substantial decrease in peel chlorophyll a content from 305 µg g^{-1} at 0 d to 70–108 µg g^{-1} and 8–41 µg g^{-1}

after 28 and 42 d, respectively (Fig. 4.2.2C). There was also a substantial reduction in peel chlorophyll b content during storage at 10 ºC and 15 ºC from the initial 135 µg g^{-1} at 0 d to about 24 µg g^{-1} and 14 µg g^{-1} after 28 and 42 d, respectively (Fig. 4.2.2C). Interestingly, fruit stored at 5 ºC, 20 ºC and 25 ºC maintained their green peel colour and there were no significant changes in CCI and chlorophyll content throughout the entire storage duration. Similar results were observed in similar experimental setups in 2014 and 2015 (Fig. 4.2.3, 4.2.4). Altogether, these findings clearly indicate that intermediate storage temperatures can accelerate peel degreening changes in satsuma mandarins.

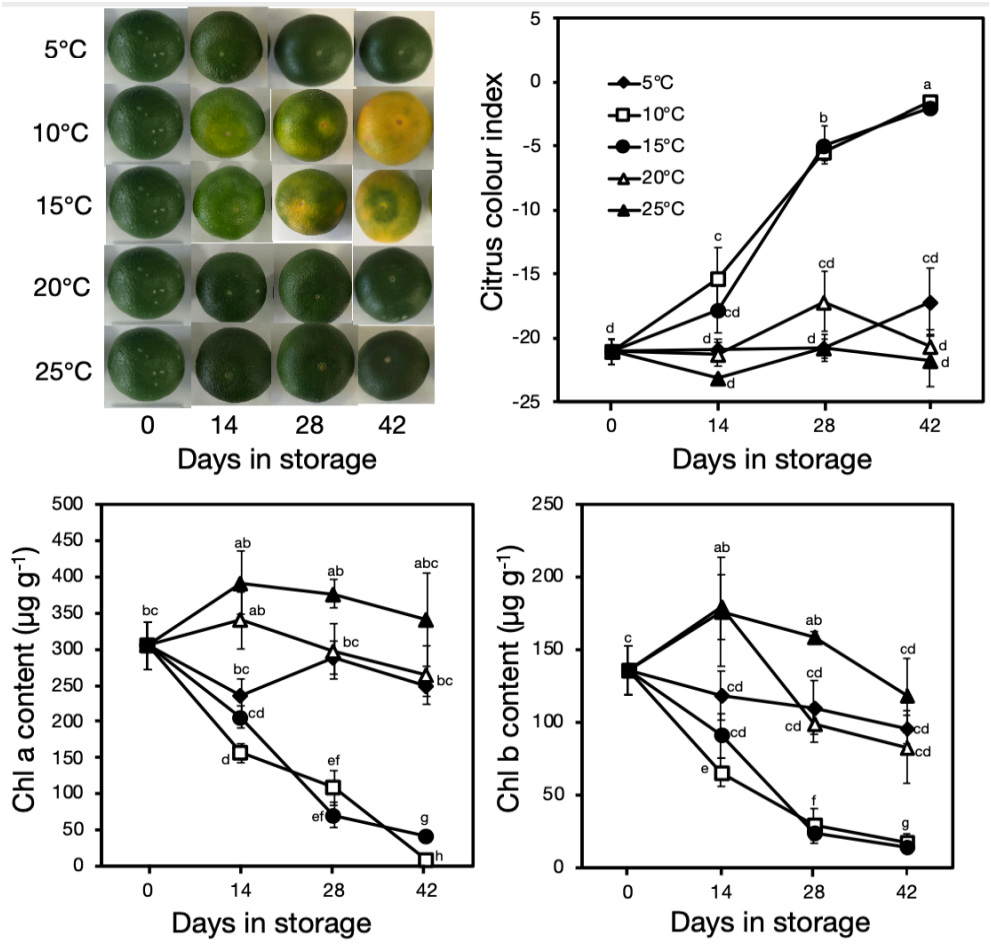


Fig. 4.2.2. Effect of storage temperature on peel degreening behaviour in satsuma mandarin fruit. (A) Appearance of fruit during storage at different temperatures. (B) Changes in citrus colour index and (C) chlorophyll content at different storage temperatures. Fruit were stored in ethylene-free chambers. Data points represent the mean (± SE) of five fruit and different letters indicate significant differences in ANOVA (Tukey's test, $p < 0.05$).

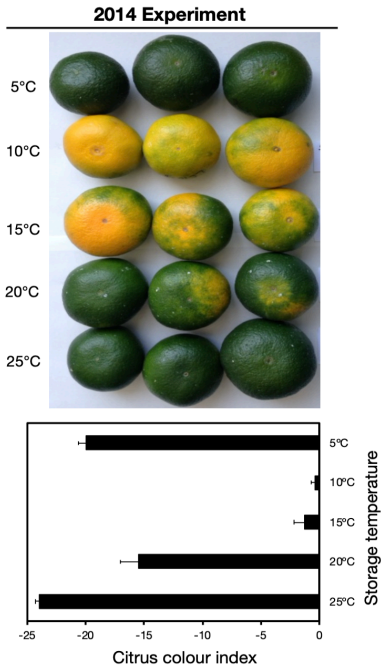


Fig. 4.2.3. Appearance and colour index of satsuma mandarin fruit after 42 d storage at 5 ºC, 10 ºC, 15 ºC, 20 ºC and 25 ºC.

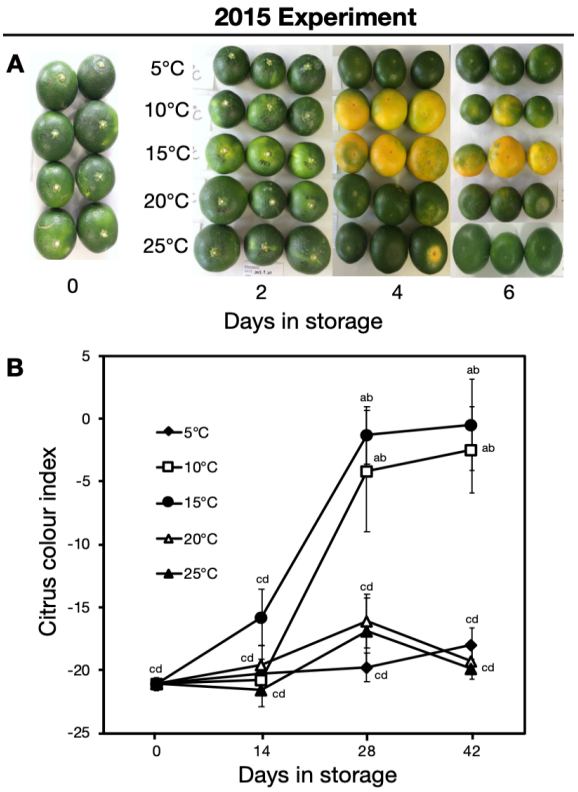


Fig. 4.2.4. Effect of storage temperature on peel degreening behaviour in satsuma mandarin fruit. (A) Appearance of fruit during storage at different temperatures. (B) Changes in citrus colour index at different storage temperatures. Fruit were stored in ethylene-free chambers. Data points represent the mean (± SE) of five fruit and different letters indicate significant differences in ANOVA (Tukey's test, $p < 0.05$).

4.2.3.3. Relationship between on-tree peel degreening and changes in ambient temperatures

Peel colour changes were further investigated during on-tree fruit maturation in relation to changes in the environmental temperature. As shown in Fig. 4.2.5A, fruit initially appeared green on Sep. 6, but they started to change their peel colour from green to yellow on Oct. 21 and finally to orange on Nov. 18 and Dec. 6. Changes in peel CCI were initially slow from -21 to -19 between Sep. 6 and Oct. 5 when the ambient temperatures were 20–24 ºC, before becoming rapid from -19 to 5 between Oct. 5 and Dec. 6 when the ambient temperatures were below 15 ºC (Fig. 4.2.5B). Peel content of both chlorophyll a and chlorophyll b also showed a similar pattern: insignificant changes between Sep. 6 and Oct. 21, before rapidly decreasing to about 10 μg g^{-1} and undetectable levels, respectively, between Oct. 21 and Nov. 18 (Fig. 4.2.5C). These observations clearly demonstrated a close relationship between peel degreening during on-tree maturation and the autumnal drops in environmental temperature.

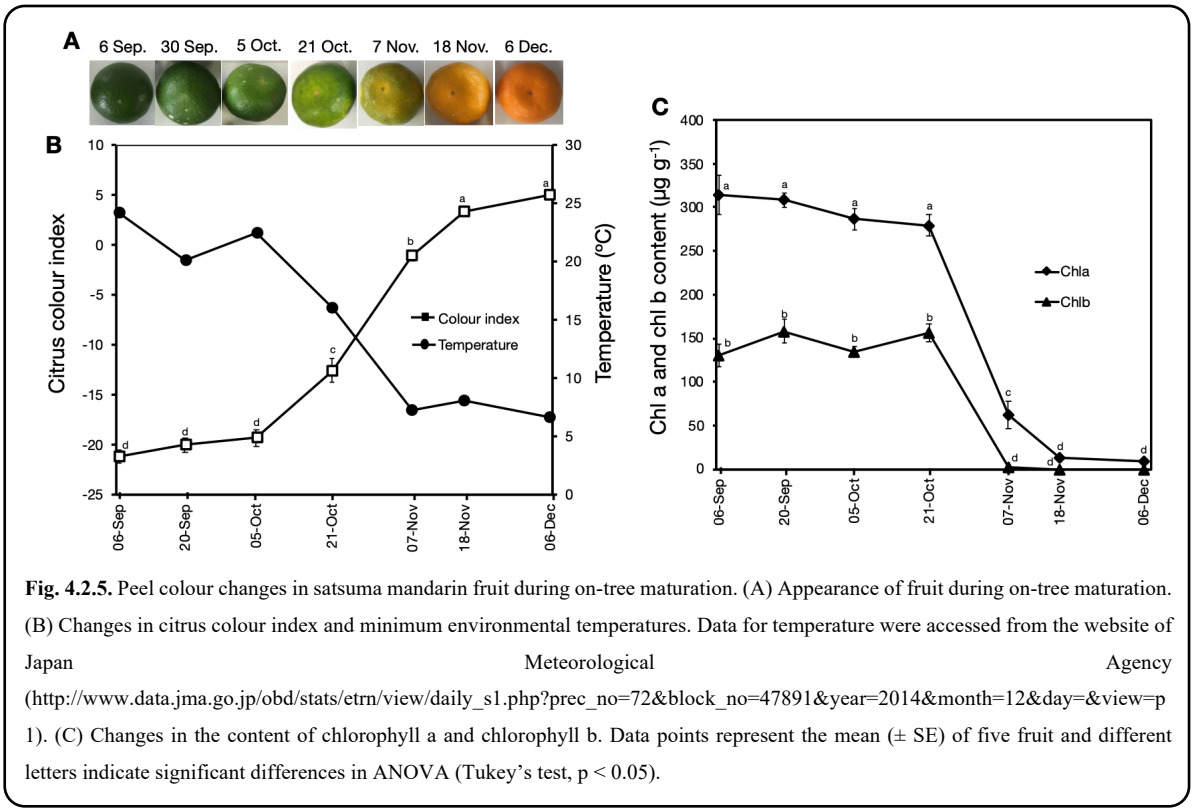


Fig. 4.2.5. Peel colour changes in satsuma mandarin fruit during on-tree maturation. (A) Appearance of fruit during on-tree maturation. (B) Changes in citrus colour index and minimum environmental temperatures. Data for temperature were accessed from the website of Japan Meteorological Agency (http://www.data.jma.go.jp/obd/stats/etrn/view/daily_s1.php?prec_no=72&block_no=47891&year=2014&month=12&day=&view=p1). (C) Changes in the content of chlorophyll a and chlorophyll b. Data points represent the mean (± SE) of five fruit and different letters indicate significant differences in ANOVA (Tukey's test, $p < 0.05$).

4.2.3.4. Transcriptome: differential expression analysis

To understand the roles of ethylene and temperature in regulation of peel degreening in satsuma mandarin fruit, we carried out a comprehensive transcriptome analysis via RNA-seq. Ethylene-induced transcriptome changes were determined by comparing 4 d propylene-treated samples with those obtained at harvest (0 d). To determine the effect of storage temperature, samples

obtained after 28 d storage at 5 ºC, 10 ºC, 15 ºC and 20 ºC were compared with those stored at 25 ºC at the same timepoint.

The above analyses revealed that 2087, 2008, 1867, 864 and 273 genes were differentially regulated by propylene, 5 ºC, 10 ºC, 15 ºC and 20 ºC respectively (Fig. 4.2.6A). The number of downregulated genes was remarkably higher in response to propylene treatment than that of upregulated genes (1686 vs. 401). Contrarily, the number of upregulated genes was considerably higher than that of downregulated genes during storage at 5 ºC (1132 vs. 876), 10 ºC (1101 vs. 766), 15 ºC (460 vs. 404) and 20 ºC (209 vs. 64). Clustering analysis showed that propylene treatment and storage at 5 ºC, 10 ºC and 15 ºC resulted in distinct gene expression patterns; expression patterns at 20 ºC were not different from those at 25 ºC (Fig. 4.2.6B). In particular, there was a distinct group of genes that were exclusively regulated by propylene treatment (ethylene-specific) while storage at 5 ºC and 10 ºC exclusively regulated another distinct gene set (low temperature-specific) (Fig. 4.2.6C). We also identified another group of genes that were regulated by either propylene (ethylene) or low temperature. Together, these findings suggested that distinct and shared pathways existed during peel degreening induced by ethylene and intermediate temperature.

4.2.3.5. RT-qPCR validation and on-tree gene expression analysis

To verify the RNA-seq data, RT-qPCR analysis was conducted using six selected genes. These included three genes in the chlorophyll degradation pathway; *CuSGR1* (*STAY GREEN 1*) whose Arabidopsis homologues have been shown to encode a Mg dechelatase (Shimoda *et al*., 2016), *CuNOL* (*NON-YELLOW COLOURING 1-LIKE*) encoding chlorophyllide b reductase and *CuACD2* (*ACCELERATED CELL DEATH 2*) which was shown to encode a red chlorophyll catabolite reductase (Mach *et al*., 2001). Additionally, two genes encoding photosystem proteins, that is, *CuCAB2* (*CHLOROPHYLL A/B BINDING PROTEIN*) and *CuLHCB2* (*LIGHT HARVESTING COMPLEX 2*) were analysed. *CuERF114* (*ETHYLENE RESPONSIVE FACTOR 114*) encodes a transcription factor.

Relative expression levels of chlorophyll degradation-related genes *CuSGR1*, *CuNOL* and *CuACD2* increased after 4 d of propylene treatment (Fig. 4.2.7A–C). Propylene treatment also increased the expression levels of *CuERF114* (Fig. 4.2.7F), whereas it caused a decrease in expression levels of *CuCAB2* and *CuLHCB2* (Fig. 4.2.7D, E). During storage, there was an increase in expression of *CuSGR1* at 5 ºC, 10 ºC, 15 ºC and 20 ºC but no significant changes

were recorded at 25 ºC (Fig. 4.2.7A). Relative expression levels of *CuSGR1* were highest at 10 ºC and 15 ºC. *CuNOL* expression increased considerably during storage at 5 ºC and 10 ºC while changes at 15 ºC, 20 ºC and 25 ºC were insignificant (Fig. 4.2.7B). The expression of *CuACD2* was elevated during storage at 10 ºC and 15 ºC with no significant changes during at 5 ºC, 20 ºC and 25 ºC (Fig. 4.2.7C). Relative expression levels of *CuCAB2* and *CuLHCB2* decreased in fruit stored at 5 ºC, 10 ºC and 15 ºC while they remained unchanged in fruit at 20 ºC and 25 ºC (Fig. 4.2.7D, E). Finally, *CuERF114* expression increased substantially in fruit stored at 5 ºC, 10 ºC and 15 ºC whereas there were no appreciable expression changes at 20 ºC and 25 ºC (Fig. 4.2.7F). Altogether, these results demonstrated that both ethylene and low/intermediate temperature could modulate the expression patterns of genes related with chlorophyll degradation and photosystem proteins as well as transcription factors.

Changes in expression levels of the above six selected genes were also examined during on-tree maturation. While the expression of *CuSGR1* initially showed minimal changes at the start of the experiment, transcript levels rose sharply by 35-fold on Oct. 21 after which they began to decrease gradually; about 14-fold increases were observed between Nov. 18 and Dec. 6 (Fig. 4.2.8A). *CuNOL*, *CuACD2* and *CuERF114* transcript levels increased steadily from Oct. 21 up until Dec. 6 (Fig. 4.2.8B, C, F). By contrast, *CuCAB2* expression remained unchanged between Sep. 6 and Oct. 21 after which it sharply decreased on Nov. 7 (Fig. 4.2.8D). There was a steady decline in *CuLHCB2* expression throughout the experimental period (Fig. 4.2.8E).

4.2.3.6. Expression patterns of ethylene-specific and low temperature-specific genes during postharvest and on-tree degreening

To gain deeper insights on the regulatory mechanisms of peel degreening at 10 ºC and 15 ºC, we examined the postharvest and on-tree expression patterns of two genes that were exclusively regulated by propylene (*CuCOPT1*: *COPPER TRANSPORTER 1* and *CuPOX-A2* : *PEROXIDASE A2*) and one gene that was exclusively regulated by low temperature (*CuERF3*) via RT-qPCR. *CuCOPT1* and *CuPOX-A2* expression significantly increased in response to propylene treatment whereas their expression levels did not change during storage (Fig. 4.2.9A, B). However, there were no measurable changes in the expression of both *CuCOPT1* and *CuPOX-A2* during on-tree maturation. On the contrary, *CuERF3* expression was not affected by propylene treatment but it increased during storage especially at 5 ºC, 10 ºC and 15 ºC with no changes at 20 ºC and 25 ºC (Fig. 4.2.9C). During on-tree peel degreening, *CuERF3* started

to increase from Oct. 21 (20-fold) and peaked on Nov. 18 (50-fold) before decreasing to about 4-fold on Dec.6. These findings demonstrated that there was a strong similarity between the peel degreening phenomenon triggered by intermediate storage temperature and that which occurs during on-tree maturation. On the other hand, there was a dissimilarity between ethylene-induced and on-tree peel degreening.

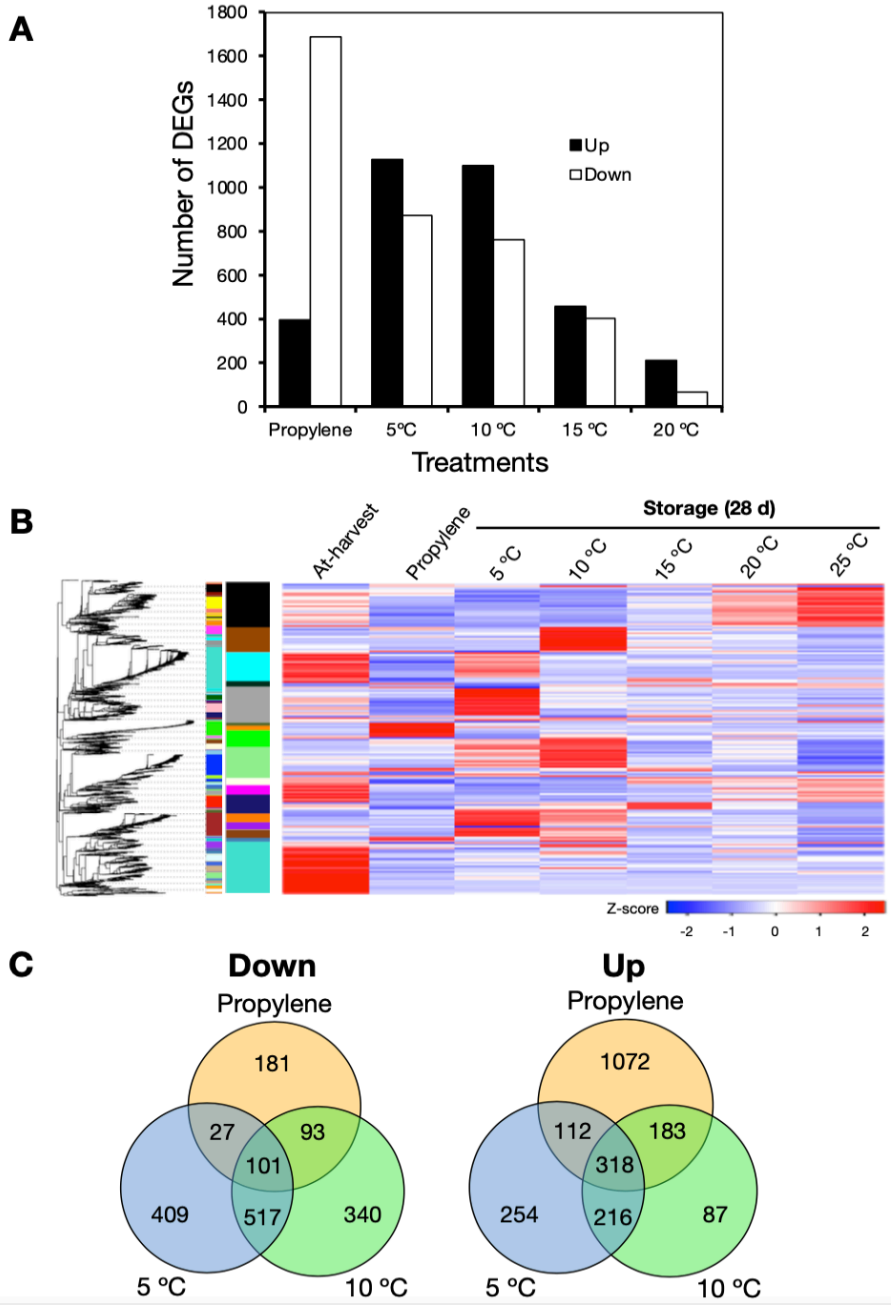


Fig. 4.2.6. Differential expression analysis in the flavedo of satsuma mandarin fruit. (A) Number of differentially expressed genes (DEGs) responding to propylene treatment and storage at 5 ºC, 10 ºC, 15 ºC and 20 ºC. Propylene-induced changes were determined by comparing with at-harvest (o d) samples. Changes at 5 ºC, 10 ºC, 15 ºC and 20 ºC were determined by comparing with 25 ºC. (B) Clustering analysis and heatmap of expression measures of DEGs detected. (C) Venn diagrams showing the number of shared and unique genes up- and down-regulated by propylene treatment and storage at 5 ºC and 10 ºC.

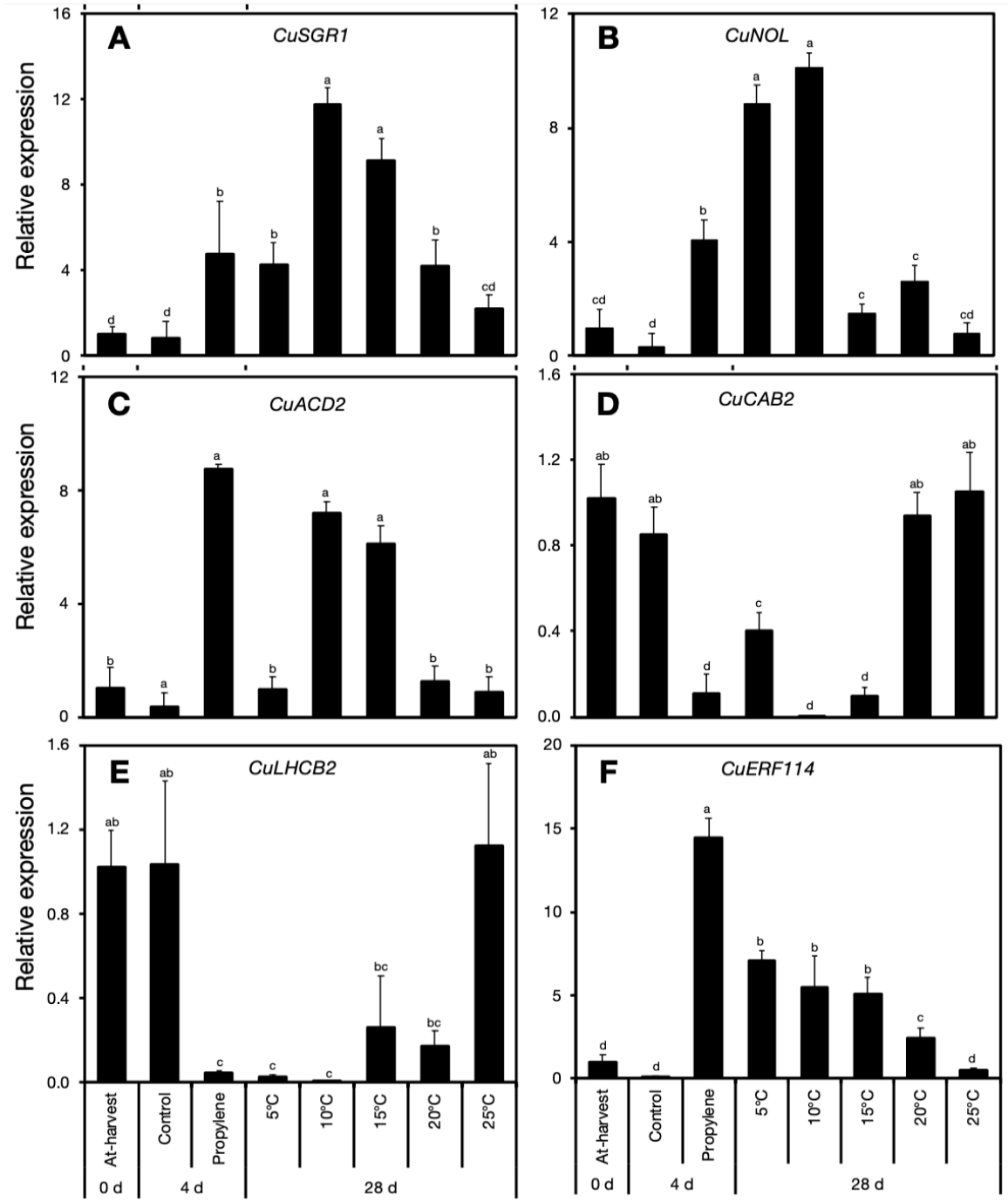


Fig. 4.2.7. RT-qPCR analysis of the expression of genes associated with chlorophyll degradation and a transcription factor in the flavedo of satsuma fruit treated with propylene for 4 d and after storage at the specified temperatures for 28 d. Data points represent the mean (± SE) of three fruit and different letters indicate significant differences in ANOVA (Tukey's test, $p < 0.05$).

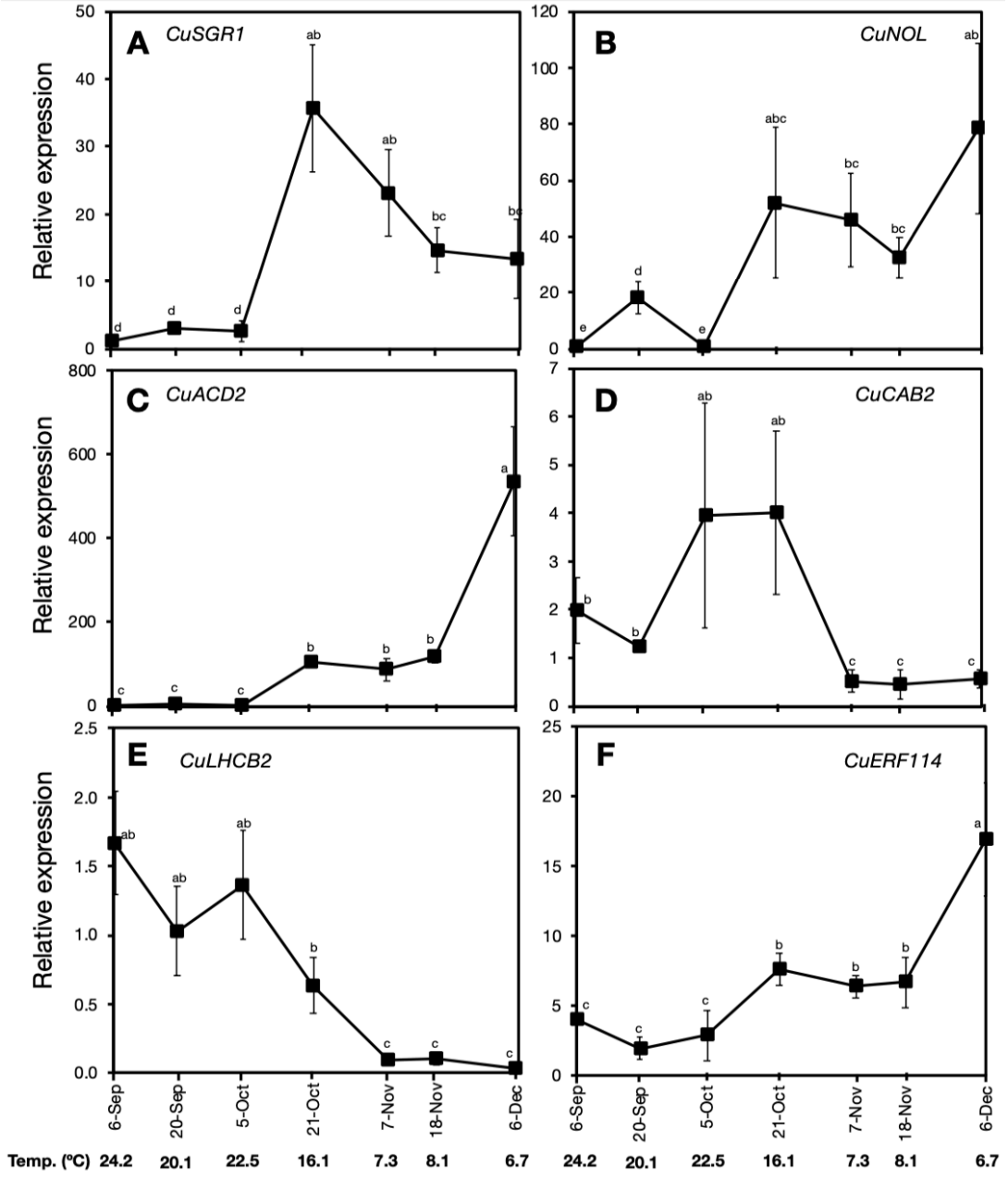


Fig. 4.2.8. Relative expression levels of genes associated with chlorophyll degradation and a transcription factor in the flavedo of satsuma mandarin fruit during on-tree peel degreening. Values below the horizontal axis indicate the average minimum environmental temperature recorded on the specified day. Data points represent the mean (± SE) of three fruit and different letters indicate significant differences in ANOVA (Tukey's test, $p < 0.05$).

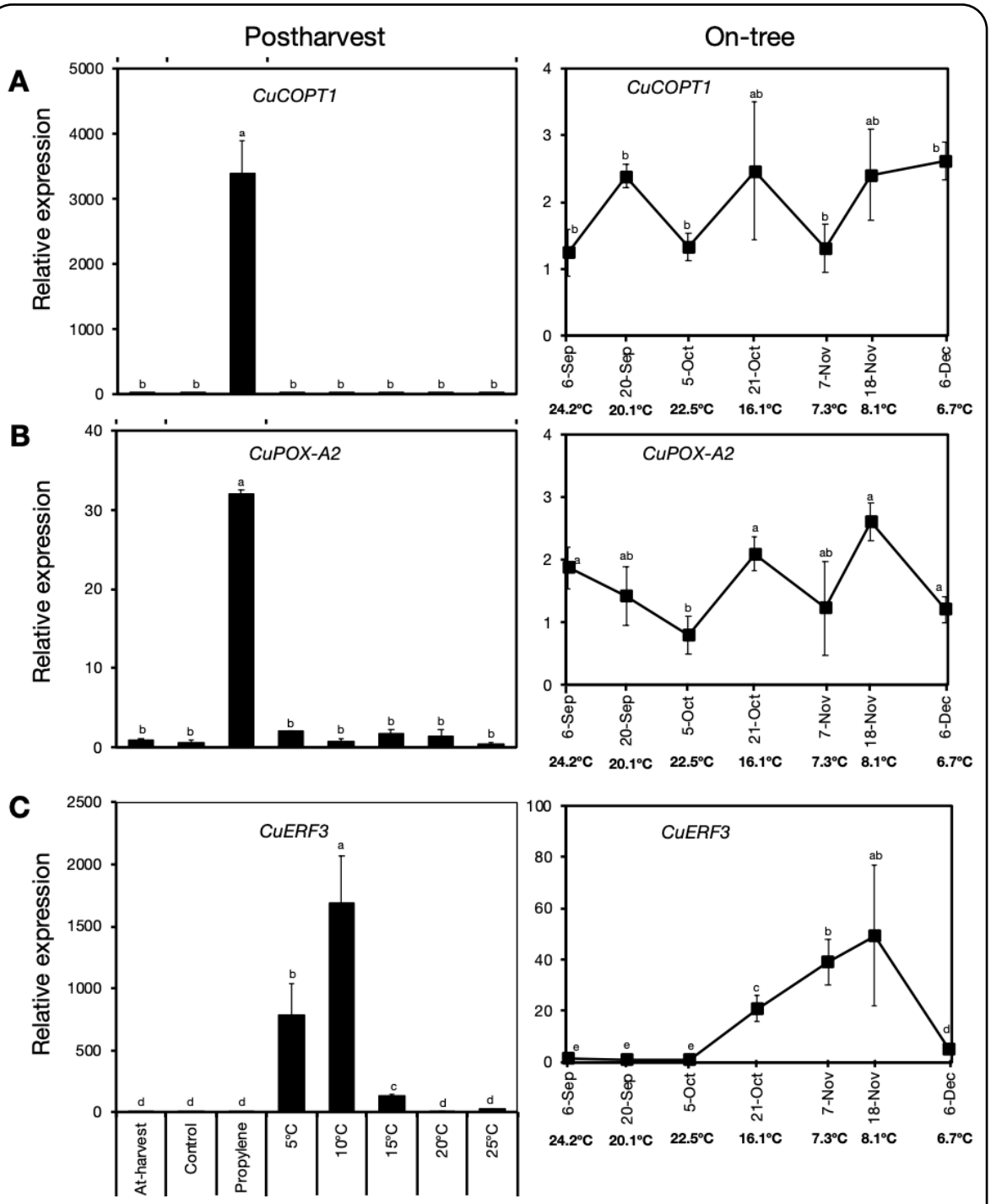


Fig. 4.2.9. Relative expression levels of ethylene-specific (A, B) and low temperature-specific (C) genes during postharvest and on-tree peel degreening in satsuma mandarin fruit. Values below the horizontal axis of on-tree graphs indicate the average minimum environmental temperature recorded on the specified day. Data points represent the mean (± SE) of three fruit and different letters indicate significant differences in ANOVA (Tukey's test, $p < 0.05$).

4.2.4. Discussion

Peel degreening is an important part of fruit ripening in citrus fruit, and it is a key determinant of quality and marketability (Jacob-Wilk *et al*., 1999; Porat, 2008). Previous studies have demonstrated that citrus peel degreening can be accelerated by either ethylene treatments (Jacob-Wilk *et al*., 1999; Porat, 2008; Shemer *et al*., 2008) or by low/intermediate temperatures (Matsumoto *et al*., 2009; Carmona *et al*., 2012a; Manera *et al*., 2012; Zhu *et al*., 2011; Tao *et al*., 2012). This is consistent with findings in the present study as both propylene treatment and storage at 10 ºC and 15 ºC triggered satsuma mandarin peel degreening (Figs. 4.2.1, 4.2.2, 4.2.3, 4.2.4). Ethylene degreening mechanisms are well established (Purvis and Barmore, 1981; Jacob-Wilk *et al*., 1999; Shemer *et al*., 2008; Yin *et al*., 2016). However, there are limited efforts towards understanding the regulatory mechanisms involved in low/intermediate temperature-induced peel degreening. A common hypothesis is that peel colour changes induced by low/intermediate temperatures are also caused by ethylene signalling (Goldschmidt *et al*., 1993; Carmona *et al*., 2012b), that is, basal levels of system I ethylene present in mature citrus fruit are physiologically active. In the present study, we provide physiological and transcriptomic evidence to suggest that low/temperature promotes citrus peel degreening independently of ethylene signalling.

Results of the RNA-seq analysis revealed that both propylene treatment and low/intermediate temperature storage triggered massive transcriptional changes during peel degreening (Fig. 4.2.6). Expression levels of chlorophyll degradation related genes such as *CuSGR1*, *CuNOL* and *CuACD2* increased in response to propylene treatment and storage at 10 ºC and 15 ºC (Fig. 4.2.7A–C), which would account for the large decrease in peel chlorophyll content (Figs. 4.2.1C, 4.2.2C). Previous studies have associated chlorophyll loss to increased expression levels of *SGR1* (Shimoda *et al*., 2016), *NOL* (Sato *et al*., 2009), and *ACD2* (Mach *et al*., 2001). Furthermore, chlorophyll degradation in response to propylene treatment and storage at 10 ºC and 15 ºC could also be attributed to decreased expression levels of *CuCAB1* and *CuLHCB2*, which are known to encode photosystem proteins. This is in agreement with Barry (2009) who postulated that disruption of pigment-protein complexes is a crucial step in the chlorophyll degradation pathway, possibly by providing free chlorophyll molecules that can easily be accessed by degradatory enzymes. In support, independent studies have demonstrated that SGR

(Mg Dechelatase) participates in chlorophyll degradation by aiding the dis-aggregation of photosystem proteins, particularly the light-harvesting chlorophyll a/b binding (CAB) complex (Jiang *et al*., 2011; Sakuraba *et al*., 2012). An earlier study also demonstrated that *CitCAB1* and *CitCAB2* expression dramatically decreased during natural and ethylene-induced peel degreening in 'Ponkan' mandarins (Peng *et al*., 2013). In the present study, increased expression levels of genes associated with transcription factors such as *CuERF114* in response to both propylene treatment and storage at 10 ºC and 15 ºC (Fig. 4.2.7F) suggests a mechanism by which chlorophyll degradation is transcriptionally regulated.

On-tree peel degreening coincided with environmental temperature drops (Fig. 4.2.5), and it was accompanied with increased expression levels of *CuSGR1*, *CuNOL*, *CuACD2* and *CuERF114* as well as decreased expression of *CuCAB2* and *CuLHCB2* (Fig. 4.2.8). To determine whether ethylene signalling was involved in these changes, we compared the on-tree expression patterns of genes exclusively induced by ethylene as well as those exclusively induced by low temperature. It is interesting that ethylene-specific genes *CuCOPT1* and *CuPOX-A2* did not show any specific changes in expression during on-tree peel degreening (Fig. 4.2.9A, B). These findings strongly suggest that ethylene signalling was non-functional during the degreening changes induced in fruit attached to the tree. It is also interesting that *CuERF3*, which showed an exclusively low temperature-dependent expression pattern, also exhibited increased transcript accumulation during on-tree degreening (Fig. 4.2.9C). It is therefore plausible to suggest that low temperature plays a role in the peel colour change events in satsuma mandarin fruit independently of ethylene.

Perhaps, a clear distinction between ethylene-induced and low temperature-induced peel degreening could be achieved by using ethylene antagonists such as 1-MCP. Our previous studies employed the use of 1-MCP to block ethylene perception and signalling, and we demonstrated that kiwifruit ripening induction by low temperature is independent of ethylene (Asiche *et al*., 2018; Mitalo *et al*., 2018a; Mitalo *et al*., 2019a; Mitalo *et al*., 2019b). In satsuma mandarins, our attempts to use 1-MCP were unsuccessful since 1-MCP treatments triggered massive climacteric-like ethylene production followed by rapid senescence (Fig. 4.2.10).

It is incredible that fruit at 5 ºC remained green throughout the storage duration (Fig. 2), despite the differential regulation of a large number of genes (Figs. 4.2.6, 4.2.7, 4.2.8, 4.2.9C). This could be due to low enzyme activity at 5 ºC, since low temperature is known to decrease

enzyme activity in plants (Jin *et al*., 2009; Yun *et al*., 2012). Another possibility could be that the translation rate might be low at 5 ºC, which could result in low levels of peel degreening-related enzymes.

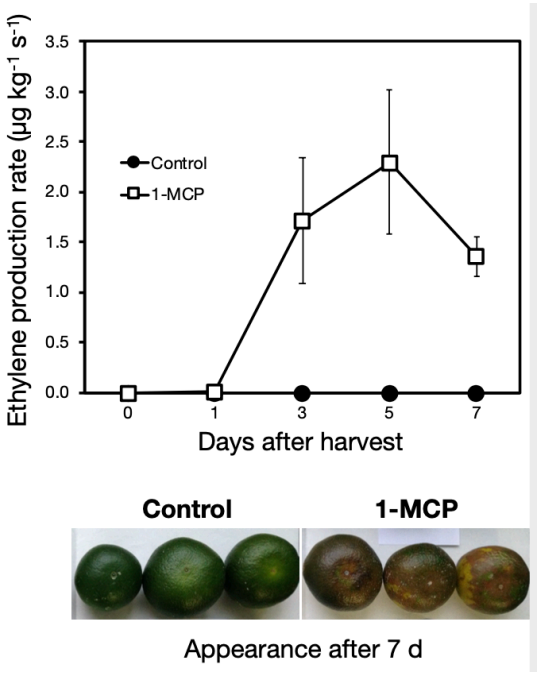


Fig. 4.2.10. Treatment with 1-MCP (2 µL L^{-1}) induced climacteric-like ethylene production and senescence in satsuma mandarin fruit.

4.2.5. Conclusion

In summary, this study demonstrates that peel degreening in satsuma mandarins can be stimulated by either ethylene or low temperature treatments. Transcriptome analysis unveiled distinct gene sets that are regulated by either stimulus. On-tree peel degreening coincided with a decrease in environmental temperature, and it was accompanied by differential expression of low temperature-regulated genes. On the other hand, strictly ethylene-dependent genes showed no changes in expression during on-tree peel degreening. Together, these findings support the hypothesis that low temperature can modulate citrus peel degreening independently of ethylene signalling.

CHAPTER 5

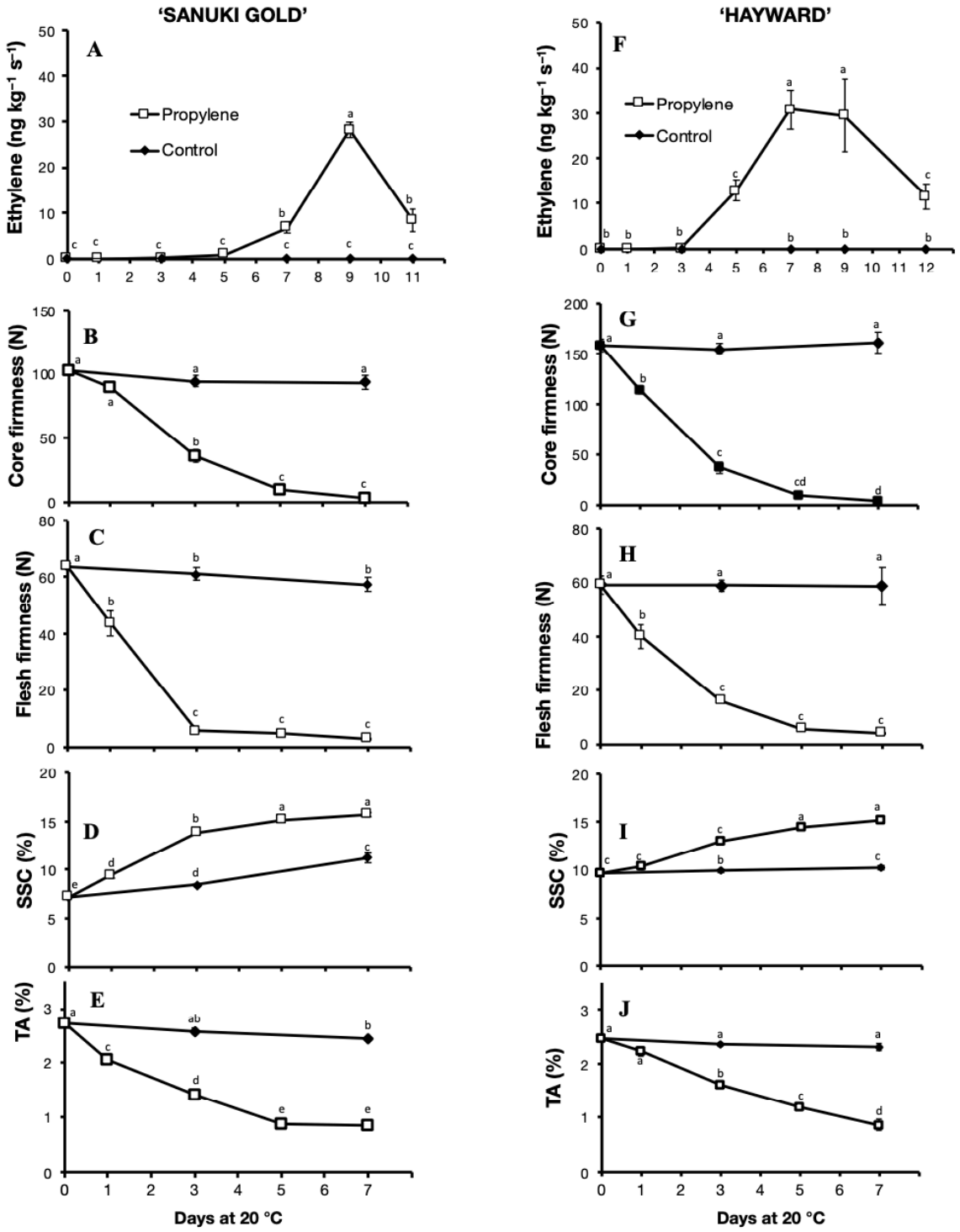
General conclusions and directions for future research

The human diet largely comprises of the consumption of fruit, which form a major source of sugars, minerals, vitamins and beneficial phytochemicals that are required for a healthy lifestyle. Fruit ripening is important as it is the process that imparts palatability to fruit. However, fruit ripening is also a leading cause (both directly and indirectly) of postharvest losses in horticultural industries. It is thus essential to improve our understanding of fruit ripening through research to improve the nutritional value of fruit while reducing postharvest losses. The need to ensure adequate and stable food supply for the ever-growing world population, coupled with the ongoing reduction of arable land, also make fruit ripening-related research relevant.

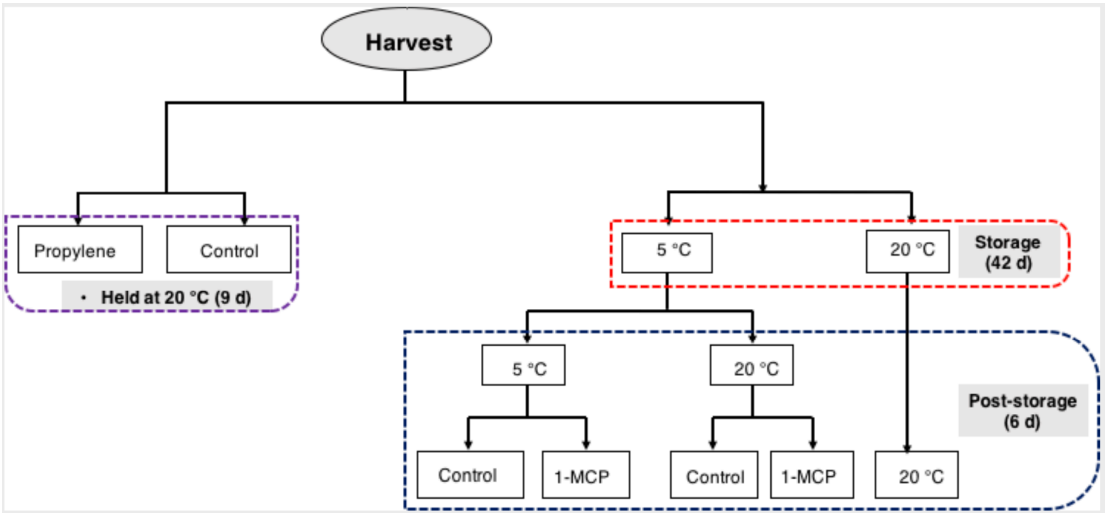
The research presented in this thesis has advanced our understanding of how fruit ripening is regulated by examining the role played by low temperature. Before this research, the common dogma in postharvest physiology has been that low temperature retards fruit ripening and hence the wide use of cold storage facilities in the postharvest handling of most fruit. Consequently, ethylene has been considered as the main culprit during postharvest storage and all the unusual ripening patterns observed in the absence of detectable ethylene are ultimately attributed to ethylene signalling. In this thesis, however, we have seen how low temperature transcriptionally regulates kiwifruit ripening independently of ethylene via a dose × time-dependent mechanism which is reflected in differences among the cultivars with regard to harvest maturity dates and postharvest storability. In citrus fruit, results of this thesis show that low temperature also transcriptionally regulates peel degreening independently of ethylene. On the other hand, research in European pears show that low temperature regulates fruit ripening by triggering system II ethylene biosynthesis and potentiating the transcriptional responses to ethylene.

A notable outcome of this research is the identification of fruit ripening-related transcription factor-encoding genes that are exclusively regulated by low temperature, such as *AcMADS2* and *AcbZIP2* in kiwifruit, *PcERF98-like* and *PcATL65* in European pears, and *ERF3* in citrus fruit. These genes are strong candidate transcriptional regulators of low temperature-modulated fruit ripening. It is recommended that future research involve a more in-depth characterisation of these genes using cutting-edge tools such as TILLING and CRISPR/Cas9 systems.

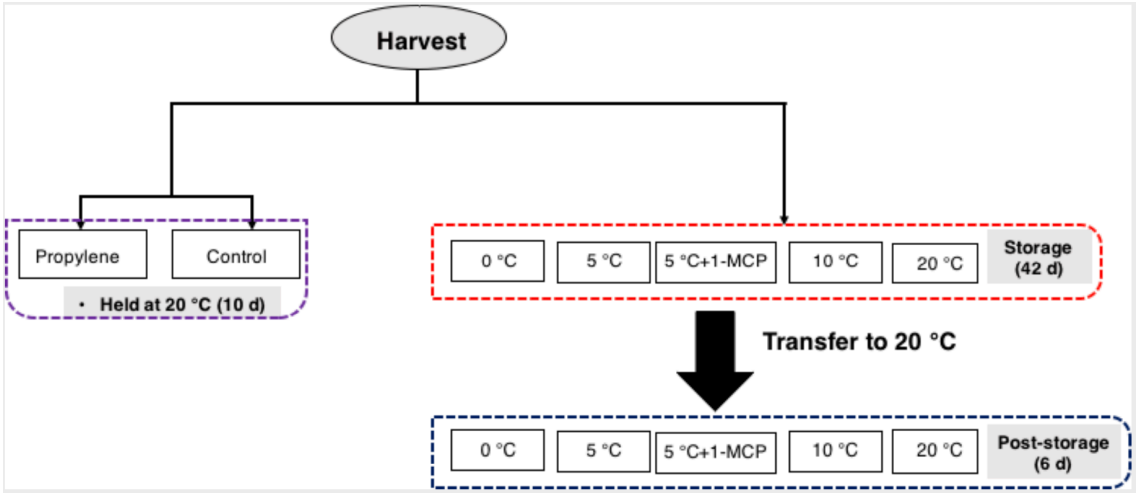
Appendices



Appendix 1. Effect of propylene (an ethylene analogue) on ethylene production and ripening characteristics of 'Sanuki Gold' (A, B, C, D, E) and 'Hayward' (F, G, H, I, J) kiwifruit (*Source*: Asiche *et al*., 2018). Fruit were exposed continuously to 5000 µL L^{-1} propylene at 20 °C. Each data set represents the mean ± SE of five independent biological replicates.



Appendix 2. A simplified diagrammatic representation of the experimental setup in the 2015 harvest season. 1-MCP treatment (2 µL L^{-1} for 12 h) was carried out just once at the end of 42 d storage.



Appendix 3. A simplified diagrammatic representation of the experimental setup in the 2016 harvest season. 1-MCP treatment (2 µLL^{-1} for 12 h) was carried out during storage on a weekly basis for a select group of fruit at 5 ºC.

Appendix 4. Liquid chromatography conditions. MeCN: methyl cyanide (acetonitrile), SA: salicylic acid.

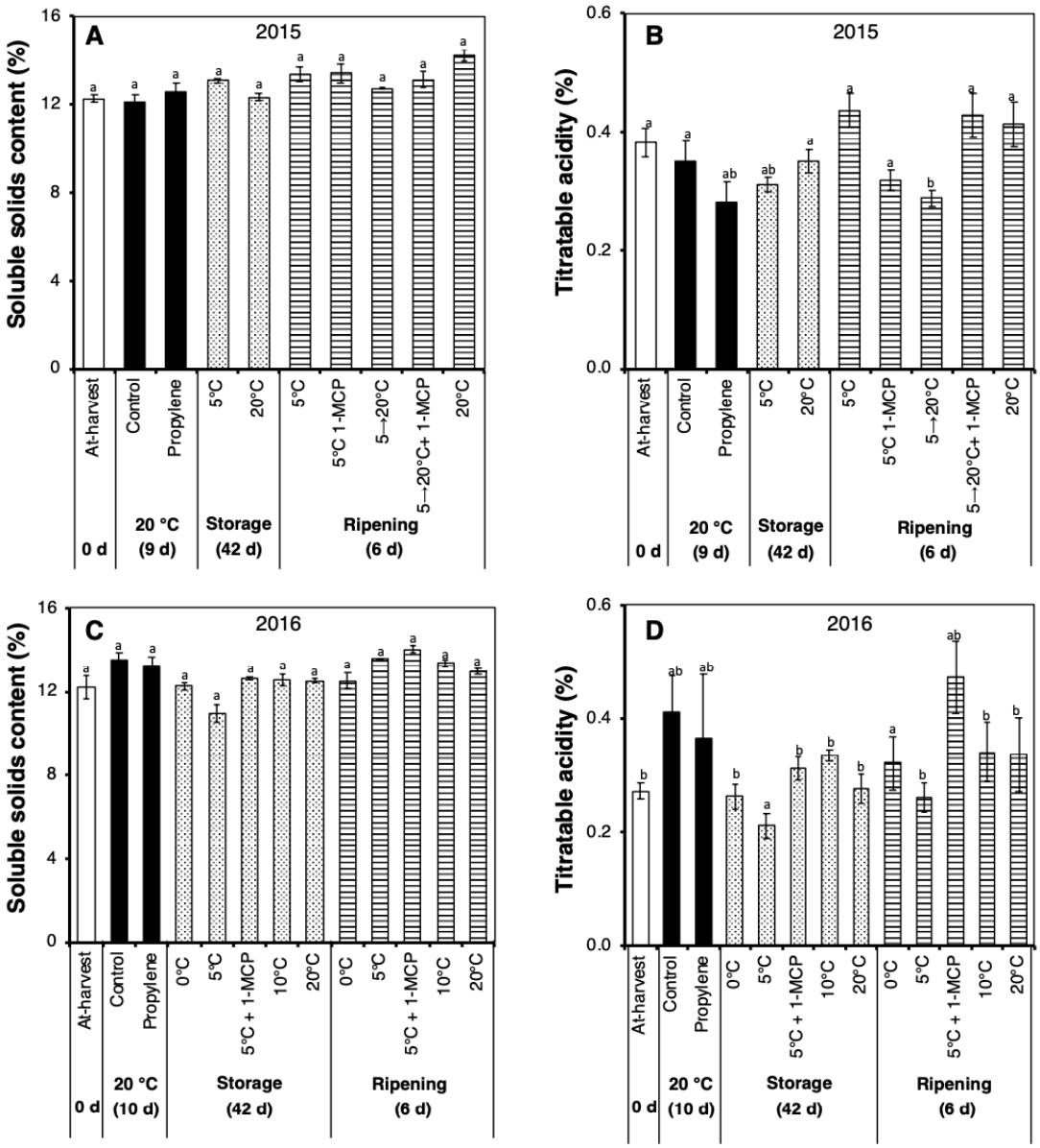
Method No.	Hormone	Solvent A	Solvent B	Gradient (Composition of solvent B)
1	Acid	Water containing 0.01 % acetic acid	MeCN containing 0.05 % acetic acid	3 to 55 % 22 min
2	CK	Water containing 0.01 % acetic acid	MeOH containing 0.2 % acetic acid	3 to 97 % 16 min
3	SA	Water containing 0.1 % formic acid	MeCN containing 0.1 % formic acid	3 to 98 % 10 min

Appendix 5. Parameters for LC-ESI-MS/MS analysis (Agilent 1260-6410). GA_1: gibberellin A_1, IAA: indole-3-acetic acid, ABA: abscisic acid, JA: jasmonic acid, GA_4: gibberellin A_4, JA-Ile: jasmonoyl-*L*-isoleucine, tZ: *trans*-zeatin, iP: N6-isopentenyladenine, SA: salicylic acid.

	LC method	Retention time on LC (min)	ESI	MS/MS transitions for quantifications (*m/z*)	Collision energy (V)	Fragmentor V
GA_1 D_2-GA_1	1	8.2	-	347/273 349/275	18	160
IAA D_2-IAA $^{13}C_6$-IAA D_7-IAA	1	9.2	+	176/130 178/132 182/136 183/135, 136, 137	15	90
ABA D_6-ABA	1	11.5	-	263/153 269/159	4	130
JA D_2-JA D_5-JA	1	13.0	-	209/59 211/59 214/62	11	135
GA_4 D_2-GA_4	1	15.4	-	331.2/257 333.2/259	20	160
JA-Ile $^{13}C_6$-JA-Ile	1	16.2	-	321.2/130 338.4/136.2	17	140
tZ D5-tZ	2	8.1	+	220.3/136.3 225.3/136.3, 137.3	12	100
iP D_6-iP	2	12	+	204.4/136.4 210.4/137.4	11	110
SA D_4-SA	3	5.3	-	137/93 141/97	15	90

Appendix 6. Oligonucleotide sequences of primers used for RT-qPCR analysis in Chapter 3

Name	Gene ID	Description	Sequence (5' to 3')	Sequence (3' to 5')
		Group I - Differentially regulated by propylene exposure without chilling		
PcACO1	PCP011683	ACC oxidase 1	GGAATTTGCAGTGGAATTGG	TGACCTTGGTCCCAAAATTC
PcPG1	PCP006771	Polygalacturonase 1	TGTAAGTGGGTCCCAAAGAG	TGGTGACATTGTTCATTTGC
PcβGAL2	PCP005049	β-Galactosidase 2	GATCTGCACAAAGCGATCAA	CATGGTGGCAGGTCATACTG
PcEXP9	PCP032946	Expansin 9	GCCTGAGTGGACGTAATAAGC	TGTAGAAGGAGAGTGTGAGATGG
PcPL18a	PCP023641	Pectate lyase 18a	GGGAACGCTATGGTGAGGAG	GACCCATAAATCGCATCAAC
PcαAMY	PCP010988	α-Amylase	GAGCTCTCGGATTGGATGAA	GATCTGGGTTCGGTTTTCC
PcGRAS2	PCP010214	GRAS family trancription factor	CAATCAACGGCCCTCTTCTC	CAATCGCTTTCTCGTACTCTCG
		Group II - Do not respond to propylene exposure; differentially regulated at low temperature; 1–MCP treaatment has no effect		
PcWAT1-like	PCP005917	Walls are thin 1-like	ACTCACACAGCCACAACAGC	AAGCATATCCAAGCCGTGAG
PcERF98-like	PCP001998	Ethylene response factor 98-like	GCAGCATCTTGAGAAAGAATCC	CTTCGATCCCACAGATTAGC
PcATL65	PCP044816	RING-H2 finger protein ATL65-like	ACCCAACTGATCAGGGACTG	GATGGTGGTGGTGATGATGA
PcMYB6-like	PCP031927	MYB family transcription factor	CTTGCTGGGAAACAAATGGT	GGACGGTTTCTCAAGTCCAA
PcGRP2-like	PCP023938	Glycine-rich protein 2-like	GTGGTGGATATGGAGGGAGA	TACCACCGCAGTTGTAGCAC
PcTCP7	PCP025373	TCP family transcription factor	CCCTTCTAAGGACCGACACA	AGAAGGAGGCGGGAGTGG
PcPSBO1	PCP031098	Photosystem II PsbO, manganese-stabilising	CTACTTCCGCTCTCGTCGTT	GGATGTTGGCTCTAGGCAGA
PcMBF1c	PCP029123	Multiprotein-bridging factor 1c-like	TTGTCAGCGTGAAGAAGCTC	CAGAACCCTCTCCATCTTCG
		Group III - Do not respond to propylene exposure; differentially regulated at low temperature; 1–MCP has a significant effect		
PcACS1	PCP011500	ACC synthase 1	AAATCTCGTCTCCGGCCTTC	AAGTTGGCAAAGCAGACACG
Pc2OGD	PCP017796	2-oxoglutarate-dependent dioxygenase	TTTCGGTCGCTACATTCCTC	AGTGCAAGGGCTTCACCAG
PcETR2	PCP002455	Ethylene receptor 2	TGTGGGAGGGTTGGTAATGT	TGCAAATGGTGAAGCTCAAG
PcEG3	PCP022047	Endo-1,4-beta-glucanase 3	GGTTGATGAAGGGAGAGCTTC	ATCTTCAACACGGTTCTTGG
PcEXPA1-like	PCP006687	Expansin A1-like	TTGCTTGTGGGTATGGAGGA	ACAAGCCCCACAGCTCAAC
PcPL18b	PCP018409	Pectate lyase 18b	CACGGACTGAACATACACGAT	CAGTTGGACAACGAGCAATG
PcSAUR21-like	PCP011953	Auxin-responsive protein SAUR21-like	GTAGTCCCGAAAGGCCATGT	TCCCAGCTCGTAAATGAGTCTA
PcMADS2	PCP013107	MADS-box related transcription factor	TTGGAGCAACAGCTTGACAC	GCAGCCTTCTCCTTCTCCTT
PcBZR1	PCP024976	Brassinazole resistant 1	CTATCTCCGCTCTGCAATCC	GCGTAAAACGGGTAATCGAA
PcActin	PCP000431	Actin, Housekeeping gene	TGGGAGAGATGGATTTGGAG	GGTGAAGGTCTCGAGCACAG



Appendix 7. Soluble solids content (A, C) and titratable acidity (B, D) of 'Passe Crassane' fruit treated with propylene (5000 µL L^{-1}) at 20 ºC for 9–10 d, and after storage at various temperatures for 42 d prior to ripening (for 6 d) at varied conditions. Values represent means (± SE) of three independent biological replications. Different letters indicate significant differences in ANOVA (Tukey test; $p < 0.05$).

Appendix 8. Primer sequences used for RT-qPCR analysis in Chapter 4.1.

Gene ID	Symbol	Fw	Rv
Ciclev10022121m.g	*ClCHYb1*	AGAGCTCTATGGCACGCTTCA	GCTGGTGATGAGACTGCAAAA
Ciclev10011841m.g	*ClPSY1*	GCACCCGGCTAGCATATCT	GAGGTGCAACTTAGGGGTGA
Ciclev10031731m.g	*ClPPH*	CCCCCGTCATTTAGAGAACA	TTGGGAAACTACCTGCATCC
Ciclev10027504m.g	*ClbHLH25*	CAACTTTGACGCTCAATCCA	CAAGACGTTGCAACTGAGGA
Ciclev10021103m.g	*ClCLH1*	ATCATCTCCATCCTCACCAC	CTGAGGAGCAACAACGATGA
Ciclev10028245m.g	*ClLCYb2a*	GTGATCATCATTGGCACTGGA	AAGTCATCGGCCAAGTTTTG
Ciclev10016280m.g	*ClLHCB2*	CTTCTCTGAAGGTGGCCTTG	CAAGTGGGTCAAAAGCACCAC
Ciclev10014639m.g	*ClNCED5*	ACCCACGTGTCCAAATTAGC	ACTTGCGCTTCCGTTTCC
Ciclev10032575m.g	*ClERF114*	CCGCTACAACTTCACAGCAA	GAGACGACGTCGCAGAAGA
Ciclev10009593m.g	*ClERF3*	GAAACAGCTGAAGACGCTGC	AGGCCATGTAGCACCTATGC

Appendix 9. List of primers used in Chapter 4.2.

Gene ID	Symbol		Sequence
Ciclev10030036m.g	*CuCOPT1*	Fw	TTCTGGGGCACAAATACTGA
		Rv	CCAATCCTGAAGGCGTACAT
Ciclev10009593m.g	*CuERF3*	Fw	GAAACAGCTGAAGACGCTGC
		Rv	AGGCCATGTAGCACCTATGC
Ciclev10008039m.g	*CuNOL*	Fw	TGCACTCGTGAGGCTATGC
		Rv	GCTGTATGCACCCCAACTTTA
Ciclev10026248m.g	*CuACD2*	Fw	CAAGTGACGCTTGCTGGATA
		Rv	CTTCCCGTTGGTACCTTCAA
Ciclev10016286m.g	*CuCAB2*	Fw	GTTGGGAGCTCTTGGATGC
		Rv	CATCAATACCACCTGGCAA
Ciclev10021651m.g	*CuSGR1*	Fw	GAGTTGAAGCAACCACAACC
		Rv	AGTCTTGGGGACAACACACA
Ciclev10032575m.g	*CuERF114*	Fw	CCGCTACAACTTCACAGCAA
		Rv	GAGACGACGTCGCAGAAGA
Ciclev10015790m.g	*CuPOX-A2*	Fw	GTGATGCATCGATTTTGCTG
		Rv	ATTACCAAAGCAACGGATCG
Ciclev10016280m.g	*CuLHCB2*	Fw	CTTCTCTGAAGGTGGCCTTG
		Rv	CAAGTGGGTCAAAAGCACCAC

Bibliography

Abeles F. (1992). *Ethylene in Plant Biology*, 2nd ed. (New York: Academic Press).

Abeles, F. B., Gahagan, H. E. (1968). Abscission: The role of ethylene, ethylene analogues, carbon dioxide, and oxygen. *Plant Physiology*, *43*, 1255–1258.

Adato, A., Mandel, T., Mintz-Oron, S. *et al*. (2009). Fruit-surface flavonoid accumulation in tomato is controlled by a SlMYB12-regulated transcriptional network. *PLoS Genetics*, *5*, e1000777. https://doi.org/10.1371/journal.pgen.1000777.

Agar, I. T., Biasi, W. V., Mitcham, E. J. (1999). Exogenous ethylene accelerates ripening responses in Bartlett pears regardless of maturity or growing region. *Postharvest Biology and Technology*, *17*, 67–78.

Agócs, A., Nagy, V., Szabó, Z., Márk, L., Ohmacht, R., Deli, J. (2007). Comparative study on the carotenoid composition of the peel and the pulp of different citrus species. *Innovative Food Science and Emerging Technologies*, *8*, 390–394.

Akagi, T., Henry, I.M., Tao, R., Comai, L. (2014). A Y-chromosome-encoded small RNA acts as a sex determinant in persimmons. *Science*, *346*, 646–650.

Alós, E., Cercós, M., Rodrigo, M. J., Zacarías, L., Talón, M. (2006). Regulation of color break in citrus fruits. Changes in pigment profiling and gene expression induced by gibberellins and nitrate, two ripening retardants. *Journal of Agricultural and Food Chemistry*, *54*, 4888–4895.

Ampomah-Dwamena, C., Thrimawithana, A. H., Dejnoprat, S., Lewis, D., Espley, R. V., Allan, A. C. (2019). A kiwifruit (*Actinidia deliciosa*) R2R3-MYB transcription factor modulates chlorophyll and carotenoid accumulation. *New Phytologist*, *221*, 309–325.

Antunes, M. D. (2007). The role of ethylene in kiwifruit ripening and senescence. *Stewart Postharvest Review*, *3*, 1–8.

Antunes, M. D. C., Pateraki, I., Kanellis, A. K., Sfakiotakis, E. M. (2000). Differential effects of low-temperature inhibition on the propylene induced autocatalysis of ethylene production, respiration and ripening of 'Hayward' kiwifruit. *The Journal of Horticultural Science and Biotechnology*, *75*, 575–580.

Arpaia, M. L., Labavitch, J. M., Greve, C., Kader, A. A. (1987). Changes in cell wall components of kiwifruit during storage in air or controlled atmospheres. *Journal of the American Society for Horticultural Science*, *112*, 474–481.

Asiche, W. O., Mitalo, O. W., Kasahara, Y., Tosa, Y., Mworia, E. G., Ushijima, K., Nakano, R., and Kubo, Y. (2017). Effect of storage temperature on fruit ripening in three kiwifruit cultivars. *The Horticulture Journal*, *86*, 403–410.

Asiche, W. O., Mitalo, O. W., Kasahara, Y., Tosa, Y., Mworia, E. G., Owino, W. O., Ushijima, K., Nakano, R., Yano, K., Kubo, Y. (2018). Comparative transcriptome analysis reveals distinct ethylene-independent regulation of ripening in response to low temperature in kiwifruit. *BMC Plant Biology*, *18*, 47. https://doi.org/10.1186/s12870-018-1264-y.

Asiche, W. O., Mworia, E. G., Oda, C., Mitalo, O. W., Owino, W. O., Ushijima, K., Nakano, R., and Kubo, Y. (2016). Extension of shelf-life by limited duration of propylene and 1-MCP treatments in three kiwifruit cultivars. *The Horticulture Journal*, *85*, 76–85.

Asif, M. H., Lakhwani, D., Pathak, S., Gupta, P., Bag, S. K., Nath, P., Trivedi, P. K. (2014). Transcriptome analysis of ripe and unripe fruit tissue of banana identifies major metabolic networks involved in fruit ripening process. *BMC Plant Biology*, *14*, 316. https://doi.org/10.1186/s12870-014-0316-1.

Atkinson, R. G., Gunaseelan, K., Wang, M. Y., Luo, L., Wang, T., Norling, C. L. (2011). Dissecting the role of climacteric ethylene in kiwifruit (*Actinidia chinensis*) ripening using a 1-aminocyclopropane-1-carboxylic acid oxidase knockdown line. *Journal of Experimental Botany*, *62*, 3821–3835.

Atkinson, R. G., Johnston, S. L., Yauk, Y., Sharma, N. N., Schröder, R. (2009). Analysis of xyloglucan endotransglucosylase/hydrolase (XTH) gene families in kiwifruit and apple. *Postharvest Biology and Technology*, *51*, 149–157.

Ballester, A. R., Molthoff, J., de Vos, R. et al. (2010). Biochemical and molecular analysis of pink tomatoes: deregulated expression of the gene encoding transcription factor SlMYB12 leads to pink tomato fruit color. *Plant Physiology*, *152*, 71–84.

Ban, Y., Honda, C., Hatsuyama, Y., Igarashi, M., Bessho, H., Moriguchi, T. (2007). Isolation and functional analysis of a MYB transcription factor gene that is a key regulator for the development of red coloration in apple skin. *Plant and Cell Physiology*, *48*, 958–970.

Barrero-Gil, J., Huertas, R., Rambla, J. L., Granell, A., Salinas, J. (2016). Tomato plants increase their tolerance to low temperature in a chilling acclimation process entailing comprehensive transcriptional and metabolic adjustments. *Plant, Cell and Environment*, *39*, 2303–2318.

Barry, C. S. (2009). The stay-green revolution: Recent progress in deciphering the mechanisms of chlorophyll degradation in higher plants. *Plant Science*, *176*, 325–333.

Barry, C. S., Giovannoni, J. J. (2007). Ethylene and fruit ripening. *Journal of Plant Growth Regulation*, *26*, 143. https://doi.org/10.1007/s00344-007-9002-y.

Barry, C. S., Llop-Tous, M. I., Grierson, D. (2000). The regulation of 1-aminocyclopropane-1-carboxylic acid synthase gene expression during the transition from system-1 to system-2 ethylene synthesis in tomato. *Plant Physiology*, *123*, 979–986.

Bemer, M., Karlova, R., Ballester, A. R., Tikunov, Y. M., Bovy, A. G., Wolters-Arts, M., Rosetto, P. B., Angenent, G. C., de Maagd, R. A. (2012). The tomato FRUITFULL homologs TDR4/FUL1 and MBP7/FUL2 regulate ethylene-independent aspects of fruit ripening. *The Plant Cell*, *24*, 4437–4451.

Boquete, E. J., Trinchero, G. D., Fraschina, A. A., Vilella, F., Sozzi, G. O. (2004). Ripening of 'Hayward' kiwifruit treated with 1-methylcyclopropene after cold storage. *Postharvest Biology and Technology*, *32*, 57–65.

Bouzayen, M., Latché, A., Nath, P., Pech, J. C. (2010). Mechanism of fruit ripening. In *Plant developmental biology-biotechnological perspectives*, E. C. Pua, M. R. Davey, eds (Heidelberg, Berlin, Germany: Springer). p. 319−339.

Bramley, P. M. (2002). Regulation of carotenoid formation during tomato fruit ripening and development. *Journal of Experimental Botany*, *53*, 2107–2113.

Brummell, D. A. (2006). Cell wall disassembly in ripening fruit. *Functional Plant Biology*, *33*, 103–119.

Burg, S. P., Burg, E. A. (1967). Molecular requirements for the biological activity of ethylene. *Plant Physiology*, *42*, 144–152.

Cao, Z. Y., Sun, L. H., Mou, R. X., Zhang, L. P., Lin, X. Y., Zhu, Z. W., Chen, M. X. (2016). Profiling of phytohormones and their major metabolites in rice using binary solid-phase extraction and liquid chromatography-triple quadrupole mass spectrometry. *Journal of Chromatography A*, *1451*, 67–74.

Cara, B., Giovannoni, J. J. (2008). Molecular biology of ethylene during tomato fruit development and maturation. *Plant Science*, *175*, 106–113.

Carmona, L., Rodrigo, M. J., Zacarías, L. (2012b). Exploring the involvement of ethylene in the regulation of color changes in citrus fruit. *Acta Horticulturae*, 934, 879–885.

Carmona, L., Zacarías, L., Rodrigo, M. J. (2012a). Stimulation of colouration and carotenoid biosynthesis during postharvest storage of 'Navelina' orange fruit at 12 ºC. *Postharvest Biology and Technology*, *74*, 108–117.

Castellarin, S. D., Pfeiffer, A., Sivilotti, P., Degan, M., Peterlunger, E., Di Gaspero, G. (2007). Transcriptional regulation of anthocyanin biosynthesis in ripening fruits of grapevine under seasonal water deficit. *Plant, Cell and Environment*, *30*, 1381–1399.

Centeno, D. C., Osorio, S., Nunes-Nesi, A. *et al*. (2011). Malate plays a crucial role in starch metabolism, ripening, and soluble solid content of tomato fruit and affects postharvest softening. *The Plant Cell*, *23*, 162–184.

Chagné, D., Crowhurst, R. N., Pindo, M. *et al*. 2014. The draft genome sequence of European pear (*Pyrus communis* L. 'Bartlett'). *PloS One*, *9*, e92644. https://doi.org/10.1371/journal.pone.0092644.

Chang, C., Kwok, S. F., Bleecker, A. B., Meyerowitz, E. M. (1993). Arabidopsis ethylene-response gene ETR1: similarity of product to two-component regulators. *Science*, *262*, 539–544.

Cheng, Y., Ahammed, G. J., Yu, J. *et al*. (2016). Putative WRKYs associated with regulation of fruit ripening revealed by detailed expression analysis of the WRKY gene family in pepper. *Scientific Reports*, *6*, 39000. https://doi.org/10.1038/srep39000.

Cherian, S., Figueroa, C. R., Nair, H. (2014). 'Movers and shakers' in the regulation of fruit ripening: a cross-dissection of climacteric versus non-climacteric fruit. *Journal of Experimental Botany*, *65*, 4705–4722.

Chung, M. Y., Vrebalov, J., Alba, R., Lee, J., McQuinn, R., Chung, J. D., Klein, P., Giovannoni, J. (2010). A tomato (*Solanum lycopersicum*) APETALA2/ERF gene, *SlAP2a*, is a negative regulator of fruit ripening. *The Plant Journal*, *64*, 936–947.

Concha, C. M., Figueroa, N. E., Poblete, L. A., Oñate, F. A., Schwab, W., Figueroa, C. R. (2013). Methyl jasmonate treatment induces changes in fruit ripening by modifying the expression of several ripening genes in *Fragaria chiloensis* fruit. *Plant Physiology and Biochemistry*, *70*, 433–444.

Conesa, A., Manera, F. C., Brotons, J. M., Fernandez-Zapata, J. C., Simón, I., Simón-Grao, S., Alfosea-Simón, M., Nicolása, J. J. M., Valverde, J. M., García-Sanchez, F. (2019). Changes in the content of chlorophylls and carotenoids in the rind of 'Fino 49' lemons during maturation and their relationship with parameters from the CIELAB color space. *Scientia Horticulturae*, *243*, 252–260.

Crisosto, C. H., Mitcham, E. J., Kader, A. A. (2000). Kiwifruit: recommendations for maintaining postharvest. https://www.ethylenecontrol.com/produce-storage/kiwifruit/. (Accessed 15 March 2018).

Crisosto, H. C., Garner, D. (2001). 1-MCP inhibits kiwifruit softening during storage. *Perishables Handling Quarterly*, *108*, 19–20.

Cruz, A. B., Bianchetti, R. E., Alves, F. R., Purgatto, E., Peres, L. E., Rossi, M., Freschi, L. (2018). Light, ethylene and auxin signalling interaction regulates carotenoid biosynthesis during tomato fruit ripening. *Frontiers in Plant Science*, *9*, 1370. https://doi.org/10.3389/fpls.2018.01370.

Cunningham, F. X., Pogson, B., Sun, Z., McDonald, K. A., DellaPenna, D., Gantt, E. (1996). Functional analysis of the beta and epsilon lycopene cyclase enzymes of Arabidopsis reveals a mechanism for control of cyclic carotenoid formation. *The Plant Cell*, *8*, 1613–1626.

Czemmel, S., Heppel, S. C., Bogs, J. (2012). R2R3-MYB transcription factors: key regulators of the flavonoid biosynthetic pathway in grapevine. *Protoplasma*, *249*, 109–118.

Defilippi, B. G., Dandekar, A. M., Kader, A. A. (2004). Impact of suppression of ethylene action or biosynthesis on flavour metabolites in apple (*Malus domestica* Borkh) fruits. *Journal of Agricultural and Food Chemistry*, *52*, 5694–5701.

Defilippi, B. G., Kader, A. A., Dandekar, A. M. (2005). Apple aroma: alcohol acyltransferase, a rate limiting step for ester biosynthesis, is regulated by ethylene. *Plant Science*, *168*, 1199–1210.
Defilippi, B. G., Manríquez, D., Luengwilai, K., González-Agüero, M. (2009). Aroma volatiles: biosynthesis and mechanisms of modulation during fruit ripening. *Advances in Botanical Research*, *50*, 1–37.
Eaks, I. L. (1970). Respiratory response, ethylene production, and response to ethylene of citrus fruit during ontogeny. *Plant Physiology*, *45*, 334–338.
El Hadi, M., Zhang, F. J., Wu, F. F., Zhou, C. H., Tao, J. (2013). Advances in fruit aroma volatile research. *Molecules*, *18*, 8200–8229.
El-Sharkawy, I., Jones, B., Gentzbittel, L., Lelièvre, J. M., Pech, J. C., Latché, A. (2004). Differential regulation of ACC synthase genes in cold-dependent and -independent ripening in pear fruit. *Plant, Cell and Environment*, *27*, 1197–210.
El-Sharkawy, I., Jones, B., Li, Z. G., Lelièvre, J. M., Pech, J. C., Latché, A. (2003). Isolation and characterization of four ethylene perception elements and their expression during ripening in pears (*Pyrus communis* L.) with/without cold requirement. *Journal of Experimental Botany*, *54*, 1615–1625.
Elitzur, T., Vrebalov, J., Giovannoni, J. J., Goldschmidt, E. E., Friedman, H. (2010). The regulation of MADS-box gene expression during ripening of banana and their regulatory interaction with ethylene. *Journal of Experimental Botany*, *61*, 1523–1535.
Englbrecht, C. C., Schoof, H., Böhm, S. (2004). Conservation, diversification and expansion of C2H2 zinc finger proteins in the *Arabidopsis thaliana* genome. *BMC Genomics*, *5*, 39. https://doi.org/10.1186/1471-2164-5-39.
Ergun, M., Huber, D. J. (2004). Suppression of ethylene perception extends shelf-life and quality of 'Sunrise Solo' papaya fruit at both pre-ripe and ripe stages of development. *European Journal of Horticultural Science*, *69*, 184–192.
Espley, R. V., Hellens, R. P., Putterill, J., Stevenson, D. E., Kutty-Amma, S., Allan, A. C. (2007). Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. *The Plant Journal*, *49*, 414–427.
Everett, K. R. (2003). The effect of low temperatures on *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* causing body rots of avocados in New Zealand. *Australasian Plant Pathology*, *32*, 441–448.
Farrow, S. C., Facchini, P. J. (2014). Functional diversity of 2-oxoglutarate/Fe (II)-dependent dioxygenases in plant metabolism. *Frontiers in Plant Science*, *5*, 524. https://doi.org/10.3389/fpls.2014.00524.
Fellman, J. K., Miller, T. W., Mattinson, D. S., Mattheis, J. P. (2000). Factors that influence biosynthesis of volatile flavour compounds in apple fruits. *HortScience*, *35*, 1026–1033.
Ferguson, A. R. (2014). Kiwifruit in the world-2014. *Acta Horticulturae*, *1096*, 33–46.
Flores, F., Ben Amor M., Jones, B., Pech, J. C., Bouzayen, M., Latche´, A., Romojaro, F. (2001). The use of ethylene-suppressed lines to assess differential sensitivity to ethylene of the various ripening pathways in Cantaloupe melons. *Physiologia Plantarum*, *113*, 128–133.
Fonseca, S., Monteiro, L., Barreiro, M. G., Pais, M. S. (2005). Expression of genes encoding cell wall modifying enzymes is induced by cold storage and reflects changes in pear fruit texture. *Journal of Experimental Botany*, *56*, 2029–2036.
Fowler, S., Thomashow, M. F. (2002). Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *The Plant Cell*, *14*, 1675–1690.
Fujii, H., Shimada, T., Sugiyama, A., Nishikawa, F., Endo, T., Nakano, M., Ikoma, S., Shimizu, T., Omura, M. (2007). Profiling ethylene-responsive genes in mature mandarin fruit using a citrus 22K oligoarray. *Plant Science*, *173*, 340–348.
Fujisawa, M., Nakano, T., Shima, Y., Ito, Y. (2013). A large-scale identification of direct targets of the tomato MADS box transcription factor RIPENING INHIBITOR reveals the regulation of fruit ripening. *The Plant Cell*, 25, 371–386.
Fukuda, T., Suezawa, K., Katagiri, T. (2007). New kiwifruit cultivar 'Sanuki Gold'. *Acta Horticulturae*, *753*, 243−246.
Gao, H. Y., Zhu, B. Z., Zhu, H. L., Zhang, Y. L., Xie, Y. H., Li, Y. C., Luo, Y. B. (2007). Effect of suppression of ethylene biosynthesis on flavour products in tomato fruits. *Russian Journal of Plant Physiology*, *54*, 80–88.
Gerasopoulos, D., Richardson, D. G. (1997). Ethylene production by `d'Anjou' pears during storage at chilling and non-chilling temperature. *HortScience*, *32*, 1092–1094.
Giovannoni, J. J. (2004). Genetic regulation of fruit development and ripening. *The Plant Cell*, *16* (Suppl 1), S170–S180.
Giovannoni, J. J. (2007). Fruit ripening mutants yield insights into ripening control. *Current Opinion in Plant Biology*, *10*, 283–289.
Giovannoni, J. J., DellaPenna, D., Bennett, A. B., Fischer, R. L. (1989). Expression of a chimeric polygalacturonase gene in transgenic rin (ripening inhibitor) tomato fruit results in polyuronide degradation but not fruit softening. *The Plant Cell*, *1*, 53–63.
Giovannoni, J. J., Noensie, E. N., Ruezinsky, D. M., Lu, X., Tracy, S. L., Ganal, M. W., Martin, G. B., Pillen, K., Albert, K., Tankslev, S. D. (1995). Molecular genetic analysis of the ripening-inhibitor and non-ripening loci of tomato: A first step in genetic map-based cloning of fruit ripening genes. *Molecular and General Genetics MGG*, *248*, 195–206.
Golden, K. D., Williams, O. J., Dunkley, H. M. (2014). Ethylene in Postharvest Technology: A Review. *Asian Journal of Biological Science*, *7*, 135–143.
Golding, J. B., Shearer, D., Wyllie, S. G., McGlasson, W. B. (1998). Application of 1-MCP and propylene to identify ethylene-dependent ripening processes in mature banana fruit. *Postharvest Biology and Technology*, *14*, 87–98.

Golding, J. B., Singh, S. P. (2017). Use of 1-MCP in the Storage Life Extension of Fruit.
Goldschmidt, E. E., Goren, R., Even-Chen, Z., Bittner, S. (1973). Increase in free and bound abscisic acid during natural and ethylene-induced senescence of citrus fruit peel. *Plant Physiology*, *51*, 879–882.
Goldschmidt, E. E., Huberman, M., Goren, R. (1993). Probing the role of endogenous ethylene in the degreening of citrus fruit with ethylene antagonists. *Plant Growth Regulation*, *12*, 325–329.
Goren, R., Mattoo, A. K., Anderson, J. D. (1984). Ethylene binding during leaf development and senescence and its inhibition by silver nitrate. *Journal of Plant Physiology*, *117*, 243–248.
Grassi, S., Piro, G., Lee, J. M., Zheng, Y., Fei, Z., Dalessandro, G., Giovannoni, J., Lenucci, M. S. (2013). Comparative genomics reveals candidate carotenoid pathway regulators of ripening watermelon fruit. *BMC Genomics*, *14*, 781. https://doi.org/10.1186/1471-2164-14-781.
Gross, K. C., Wang, C. Y., Saltveit, M. E. (Eds.). (2016). *The commercial storage of fruits, vegetables, and florist and nursery stocks*. United States Department of Agriculture, Agricultural Research Service.
Günther, C. S., Chervin, C., Marsh, K. B., Newcomb, R. D., Souleyre, E. J. (2011). Characterization of two alcohol acyltransferases from kiwifruit (*Actinidia* spp.) reveals distinct substrate preferences. *Phytochemistry*, *72*, 700–710.
Günther, C. S., Marsh, K. B., Winz, R. A., Harker, R. F., Wohlers, M. W., White, A., and Goddard, M. R. (2015). The impact of cold storage and ethylene on volatile ester production and aroma perception in 'Hort16A' kiwifruit. *Food Chem*istry, *169*, 5–12.
Guo, Y. F., Shan, W., Liang, S. M., Wu, C. J., Wei, W., Chen, J. Y., Lu, W. J., Kuang, J. F. (2019). MaBZR1/2 act as transcriptional repressors of ethylene biosynthetic genes in banana fruit. *Physiologia Plantarum*, *165*, 555–568.
Guo, Z. H., Shu, W. S., Cheng, H. Y., Wang, G. M., Qi, K. J., Zhang, S. L., Gu, C. (2018). Expression analysis of TCP genes in peach reveals an involvement of *PpTCP.A2* in ethylene biosynthesis during fruit ripening. *Plant Molecular Biology Reporter*, *36*, 588–595.
Gupta, A., Hisano, H., Hojo, Y., Matsuura, T., Ikeda, Y., Mori, I. C., Snthil-Kumar, M. (2017). Global profiling of phytohormone dynamics during combined drought and pathogen stress in Arabidopsis thaliana reveals ABA and JA as major regulators. Scientific Reports 7, 4017. https://doi.org/10.1038/s41598-017-03907-2.
Han, Y. C., Fu, C. C., Kuang, J. F., Chen, J. Y., Lu, W. J. (2016). Two banana fruit ripening-related C2H2 zinc finger proteins are transcriptional repressors of ethylene biosynthetic genes. *Postharvest Biology and Technology*, *116*, 8–15.
Hao, P. P., Wang, G. M., Cheng, H. Y., Ke, Y. Q., Qi, K. J., Gu, C., Zhang, S. L. (2018). Transcriptome analysis unravels an ethylene response factor involved in regulating fruit ripening in pear. *Physiologia Plantarum*, *163*, 124–135.
Hao, Y., Hu, G., Breitel, D., Liu, M., Mila, I., Frasse, P., Fu, Y., Aharoni, A., Bouzayen, M., Zouine, M. (2015). Auxin response factor SlARF2 is an essential component of the regulatory mechanism controlling fruit ripening in tomato. *PLoS Genetics*, *11*, e1005649. https://doi.org/10.1371/journal.pgen.1005649.
Hardenburg, R. E., Watada, A. E., Wang, C. Y. (1986). *The commercial storage of fruits, vegetables, and florist and nursery stocks*. Washington: USDA Agriculture Handbook, 66.
Hewett, E. W., Kim, H. O., Lallu, N. (1999). Postharvest physiology of kiwifruit: the challenges ahead. *Acta Horticulturae*, *498*, 203–216.
Hiwasa, K., Kinugasa, Y., Amano, S., Hashimoto, A., Nakano, R., Inaba, A., Kubo, Y. (2003). Ethylene is required for both the initiation and progression of softening in pear (*Pyrus communis* L.) fruit. *Journal of Experimental Botany*, 54, 771–779.
Hoeberichts, F. A., Van Der Plas, L. H., Woltering, E. J. (2002). Ethylene perception is required for the expression of tomato ripening-related genes and associated physiological changes even at advanced stages of ripening. *Postharvest Biology and Technology*, *26*, 125–133.
Hörtensteiner, S. (2006). Chlorophyll degradation during senescence. *Annual Review of Plant Biology*, *57*, 55–77.
Hu, W., Wang, L., Tie, W., Yan, Y., Ding, Z., Liu, J., Li, M., Peng, M., Xu, P., Jin, Z. (2016). Genome-wide analyses of the bZIP family reveal their involvement in the development, ripening and abiotic stress response in banana. *Scientific Reports*, *6*, 30203. https://doi.org/10.1038/srep30203.
Hu, X., Kuang, S., Zhang, A. D., Zhang, W. S., Chen, M. J., Yin, X. R., Chen, K. S. (2016). Characterization of starch degradation related genes in postharvest kiwifruit. *International Journal of Molecular Sciences*, *17*, 2112. https://doi.org/10.3390/ijms17122112.
Hua, J., Meyerowitz, E. M. (1998). Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. *Cell*, *94*, 261–271.
Huang, W., Xian, Z., Kang, X., Tang, N., Li, Z. (2015). Genome-wide identification, phylogeny and expression analysis of GRAS gene family in tomato. *BMC Plant Biology*, *15*, 209. https://doi.org/10.1186/s12870-015-0590-6.
Iglesias, D. J., Cercós, M., Colmenero-Flores, J. M. *et al*. 2007. Physiology of citrus fruiting. *Brazilian Journal of Plant Physiology*, *19*, 333–362.
Ikoma, Y., Yano, M., Ogawa, K., Yoshioka, T., Xu, Z. C., Hisada, S., Omura, M., Moriguchi, T. (1996). Isolation and evaluation of RNA from polysaccharide-rich tissues in fruit quality by cDNA library construction and RT-PCR. *Journal of the Japanese Society for Horticultural Science*, *64*, 809–814.

Ilina, N., Alem, H. J., Pagano, E. A., Sozzi, G. O. (2010). Suppression of ethylene perception after exposure to cooling conditions delays the progress of softening in 'Hayward' kiwifruit. *Postharvest Biology and Technology*, *55*, 160–168.
Inaba, A., Liu, X., Yokotani, N., Yamane, M., Lu, W. J., Nakano, R., Kubo, Y. (2007). Differential feedback regulation of ethylene biosynthesis in pulp and peel tissues of banana fruit. *Journal of Experimental Botany*, *58*, 1047–1057.
Ireland, H. S., Gunaseelan, K., Muddumage, R., Tacken, E.J., Putterill, J., Johnston, J.W., Schaffer, R.J., 2014. Ethylene regulates apple (*Malus× domestica*) fruit softening through a dose× time-dependent mechanism and through differential sensitivities and dependencies of cell wall-modifying genes. *Plant and Cell Physiology*, *55*, 1005–1016.
Ireland, H. S., Yao, J. L., Tomes, S., Sutherland, P. W., Nieuwenhuizen, N., Gunaseelan, K., Winz, R. A., David, K. M., Schaffer, R. J. (2013). Apple SEPALLATA1/2-like genes control fruit flesh development and ripening. *The Plant Journal*, *73*, 1044–1056.
Itai, A., Kawata, T., Tanabe, K., Tamura, F., Uchiyama, M., Tomomitsu, M., Shiraiwa, N. (1999). Identification of 1-aminocyclopropane-1-carboxylic acid synthase genes controlling the ethylene level of ripening fruit in Japanese pear (*Pyrus pyrifolia* Nakai). *Molecular and General Genetics*, *261*, 42–49.
Itkin, M., Seybold, H., Breitel, D., Rogachev, I., Meir, S., Aharoni, A. (2009). TOMATO AGAMOUS-LIKE 1 is a component of the fruit ripening regulatory network. *The Plant Journal*, *60*, 1081–1095.
Ito, Y., Nishizawa-Yokoi, A., Endo, M., Mikami, M., Shima, Y., Nakamura, N., Kotake-Nara, E., Kawasaki, S., Toki, S. (2017). Re-evaluation of the *rin* mutation and the role of RIN in the induction of tomato ripening. *Nature plants*, *3*, 866–874. https://doi.org/10.1038/s41477-017-0041-5.
Jabbar, A., East, A. R. (2016). Quantifying the ethylene induced softening and low temperature breakdown of 'Hayward' kiwifruit in storage. *Postharvest Biology and Technology*, *113*, 87–94.
Jacob-Wilk, D., Holland, D., Goldschmidt, E. E., Riov, J., Eyal, Y. (1999). Chlorophyll breakdown by chlorophyllase: isolation and functional expression of the *Chlase1* gene from ethylene-treated citrus fruit and its regulation during development. *The Plant Journal*, *20*, 653–661.
Jeong, J., Huber, D. J. (2004). Suppression of avocado (*Persea americana* Mill.) fruit softening and changes in cell wall matrix polysaccharides and enzyme activities: differential responses to 1-MCP and delayed ethylene application. *Journal of the American Society for Horticultural Science*, *129*, 752–759.
Jiang, H., Chen, Y., Li, M., Xu, X., Wu, G. (2011). Overexpression of SGR results in oxidative stress and lesion-mimic cell death in rice seedlings. *Journal of Integrative Plant Biology*, *53*, 375–387.
Jiang, Y., Joyce, D. C., Macnish, A. J. (1999). Extension of the shelf life of banana fruit by 1-methylcyclopropene in combination with polyethylene bags. *Postharvest Biology and Technology*, 16, 187–193.
Jin, W.W., Xu, C. J., Li, X., Zhang, B., Wang, P., Allan, A. C., Chen, K. S. (2009). Expression of ROP/RAC GTPase genes in postharvest loquat fruit in association with senescence and cold regulated lignification, *Postharvest Biology and Technology*, *54*, 9–14.
Jobling, J., McGlasson, W. B., Dilley, D. R. (1991). Induction of ethylene synthesizing competency in Granny Smith apples by exposure to low temperature in air. *Postharvest Biology and Technology*, *1*, 111–118.
Johansson, N., Persson, K. O., Larsson, C., Norbeck, J. (2014). Comparative sequence analysis and mutagenesis of Ethylene Forming Enzyme (EFE) 2-oxoglutarate/Fe (II)-dependent dioxygenase homologs. *BMC Biochemistry*, *15*, 22. https://doi.org/10.1186/1471-2091-15-22.
Jomori, M. L. L., Kluge, R. A., Jacomino, A. P. (2003). Cold storage of 'Tahiti' lime treated with 1-methylcyclopropene. *Scientia Agricola*, *60*, 785–788.
Ju, C., Chang, C. (2015). Mechanistic insights in ethylene perception and signal transduction. *Plant Physiology*, *169*, 85–95.
Kamiyoshihara, Y., Tieman, D. M., Huber, D. J., Klee, H. J. (2012). Ligand-induced alterations in the phosphorylation state of ethylene receptors in tomato fruit. *Plant Physiology*, *160*, 488–497.
Karlova, R., Chapman, N., David, K., Angenent, G. C., Seymour, G. B., de Maagd, R. A. (2014). Transcriptional control of fleshy fruit development and ripening. *Journal of Experimental Botany*, *65*, 4527–4541.
Kato, M. (2012). Mechanism of carotenoid accumulation in citrus fruit. *Journal of the Japanese Society for Horticultural Science*, *81*, 219–233.
Kato, M., Ikoma, Y., Matsumoto, H., Sugiura, M., Hyodo, H., Yano, M. (2004). Accumulation of carotenoids and expression of carotenoid biosynthetic genes during maturation in citrus fruit. *Plant Physiology*, *134*, 824–837.
Kato, M., Matsumoto, H., Ikoma, Y., Okuda, H., Yano, M. (2006). The role of carotenoid cleavage dioxygenases in the regulation of carotenoid profiles during maturation in citrus fruit. *Journal of Experimental Botany*, *57*, 2153–2164.
Katz, E., Lagunes, P. M., Riov, J., Weiss, D., Goldschmidt, E. E. (2004). Molecular and physiological evidence suggests the existence of a system II-like pathway of ethylene production in non-climacteric citrus fruit. *Planta*, *219*, 243–252.
Kende, H. (1993). Ethylene biosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology*, *44*, 283–307.
Kevany, B. M., Tieman, D. M., Taylor, M. G., Cin, V. D., Klee, H. J. (2007). Ethylene receptor degradation controls the timing of ripening in tomato fruit. *The Plant Journal*, *51*, 458–467.
Khan, N. A., Khan, M. I. R., Ferrante, A., Poor, P. (2017). Ethylene: a key regulatory molecule in plants. *Frontiers in Plant Science*, 8, 1782. https://doi.org/10.3389/fpls.2017.01782.

Kim, H. O., Hewett, E. W., Lallu, N. (1999). The role of ethylene in kiwifruit softening. *Acta Horticulturae*, *498*, 255–262.
Kim, H. Y., Farcuh, M., Cohen, Y., Crisosto, C., Sadka, A., Blumwald, E. (2015). Non-climacteric ripening and sorbitol homeostasis in plum fruits. *Plant Science*, *231*, 30–39.
Kinugawa, M. (2000). Studies on infection source and control of kiwifruit ripe rot. The role of old bark as infection source. *Bulletin of Kagawa Prefecture Agricultural Experiment Station*, *52*, 41–54.
Klee, H. J. (2002). Control of ethylene-mediated processes in tomato at the level of receptors. *Journal of Experimental Botany*, *53*, 2057–2063.
Klee, H. J. (2004). Ethylene signal transduction. Moving beyond Arabidopsis. *Plant Physiology*, *135*, 660–667.
Klee, H. J., Giovannoni, J. J. (2011). Genetics and control of tomato fruit ripening and quality attributes. *Annual Review of Genetics*, *45*, 41–59.
Koh, Y. J., Hur, J. S., Jung, J. S. (2005). Postharvest fruit rots of kiwifruit (*Actinidia deliciosa*) in Korea. *New Zealand Journal of Crop and Horticultural Science*, *33*, 303–310.
Kou, X., Liu, C., Han, L., Wang, S., Xue, Z. (2016). NAC transcription factors play an important role in ethylene biosynthesis, reception and signalling of tomato fruit ripening. *Molecular Genetics and Genomics*, *291*, 1205–1217.
Koukouranas, A., Sfakiotakis, E. (2007). Effect of 1-MCP pre-storage treatment on ethylene and CO_2 production and quality of 'Hayward' kiwifruit during shelf-life after short medium- and long-term cold storage. *Postharvest Biology and Technology*, *46*, 174–180.
Kumar, R., Khurana, A., Sharma, A. K. (2014). Role of plant hormones and their interplay in development and ripening of fleshy fruits. *Journal of Experimental Botany*, *65*, 4561–4575.
Lafuente, M. T., Zacarias, L., Martínez-Téllez, M. A., Sanchez-Ballesta, M. T., Dupille, E. (2001). Phenylalanine ammonia-lyase as related to ethylene in the development of chilling symptoms during cold storage of citrus fruits. *Journal of Agricultural and Food Chemistry*, *49*, 6020–6025.
Lallu, N. (1995). Low temperature breakdown in kiwifruit. *Acta Horticulturae*, *444*, 579–585.
Langfelder, P., Horvath, S. (2008). WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics*, *9*, 559. https://doi.org/10.1186/1471-2105-9-559.
Lashbrook, C. C., Tieman, D. M., Klee, H. J. (1998). Differential regulation of the tomato *ETR* gene family throughout plant development. *The Plant Journal*, *15*, 243–252.
Laura, A., Alvarez-Parrilla, E., Gonzalez-Aguilar, G. A. (Eds.). (2009). *Fruit and vegetable phytochemicals: Chemistry, nutritional value and stability*. John Wiley & Sons.
Lelièvre, J. M., Latchè, A., Jones, B., Bouzayen, M., Pech, J. C. (1997a). Ethylene and fruit ripening. *Physiologia Plantarum*, *101*, 727–739.
Lelièvre, J. M., Tichit, L., Dao, P., Fillion, L., Nam, Y., Pech, J. C., Latchè, A. (1997b). Effects of chilling on the expression of ethylene biosynthetic genes in Passe-Crassane pear (*Pyrus communis* L.) fruits. *Plant Molecular Biology*, *33*, 847–855.
Lelievre, J. M., Tichit, L., Fillion, L., Larrigaudiere, C., Vendrell, M., Pech, J. C. (1995). Cold-induced accumulation of 1-aminocyclopropane 1-carboxylate oxidase protein in Granny Smith apples. *Postharvest Biology and Technology*, *5*, 11–17.
Li, H., Suo, J., Han, Y., Liang, C., Jin, M., Zhang, Z., Rao, J. (2017). The effect of 1-methylcyclopropene, methyl jasmonate and methyl salicylate on lignin accumulation and gene expression in postharvest 'Xuxiang' kiwifruit during cold storage. *Postharvest Biology and Technology*, *124*, 107–118.
Li, L., Lichter, A., Chalupowicz, D., Gamrasni, D., Goldberg, T., Nerya, O., Ben-Arie, R., Porat, R. (2016). Effects of the ethylene-action inhibitor 1-methylcyclopropene on postharvest quality of non-climacteric fruit crops. *Postharvest Biology and Technology*, *111*, 322–329.
Li, S. J., Xie, X. L., Liu, S. C., Chen, K. S., Yin, X. R. 2019. Auto-and mutual-regulation between two CitERFs contribute to ethylene-induced citrus fruit degreening. *Food Chemistry*, *299*, 125163. https://doi.org/10.1016/j.foodchem.2019.125163.
Li, T., Jiang, Z., Zhang, L., Tan, D., Wei, Y., Yuan, H., Li, T., Wang, A. (2016). Apple (*Malus domestica*) MdERF2 negatively affects ethylene biosynthesis during fruit ripening by suppressing *MdACS1* transcription. *The Plant Journal*, *88*, 735–748.
Li, T., Xu, Y., Zhang, L., Ji, Y., Tan, D., Yuan, H., Wang, A. (2017). The jasmonate-activated transcription factor MdMYC2 regulates ETHYLENE RESPONSE FACTOR and ethylene biosynthetic genes to promote ethylene biosynthesis during apple fruit ripening. *The Plant Cell*, *29*, 1316–1334.
Lin, Z., Hong, Y., Yin, M., Li, C., Zhang, K., Grierson, D. (2008). A tomato HD-Zip homeobox protein, LeHB-1, plays an important role in floral organogenesis and ripening. *The Plant Journal*, *55*, 301–310.
Lisec, J., Schauer, N., Kopka, J., Willmitzer, L., Fernie, A. R. (2006). Gas chromatography mass spectrometry-based metabolite profiling in plants. *Nature Protocols*, *1*, 387–396.
Liu, L., Wei, J., Zhang, M., Zhang, L., Li, C., Wang, Q. (2012). Ethylene independent induction of lycopene biosynthesis in tomato fruits by jasmonates. *Journal of Experimental Botany*, *63*, 5751–5761.
Liu, M., Gomes, B. L., Mila, I., Purgatto, E., Peres, L. E., Frasse, P., Maza, E., Zouine, M., Roustan, J. P., Bouzayen, M., Pirrello, J. (2016). Comprehensive profiling of ethylene response factor expression identifies ripening-associated ERF genes and their link to key regulators of fruit ripening in tomato. *Plant Physiology*, *170*, 1732–1744.

Lovisetto, A., Guzzo, F., Tadiello, A., Confortin, E., Pavanello, A., Botton, A., Casadoro, G. (2013). Characterization of a bZIP gene highly expressed during ripening of the peach fruit. *Plant Physiology and Biochemistry*, *70*, 462–470.

Lyons, J. M. (1973). Chilling injury in plants. *Annual Review of Plant Physiology*, *24*, 445–466.

Mach, J. M., Castillo, A. R., Hoogstraten, R., Greenberg, J. T. (2001). The Arabidopsis-accelerated cell death gene *ACD2* encodes red chlorophyll catabolite reductase and suppresses the spread of disease symptoms. *Proceedings of the National Academy of Sciences*, *98*, 771–776.

Manera, F. J., Brotons, J. M., Conesa, A., Porras, I. (2012). Influence of temperature on the beginning of degreening in lemon peel. *Scientia Horticulturae*, *145*, 34–38.

Manera, F. J., Brotons, J. M., Conesa, A., Porras, I. (2013). Relation between temperature and the beginning of peel color change in grapefruit (*Citrus paradisi* Macf.). *Scientia Horticulturae*, *160*, 292–299.

Manning, K., Tör, M., Poole, M., Hong, Y., Thompson, A. J., King, G. J., Giovannoni, J. J., Seymour, G. B. (2006). A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. *Nature Genetics*, *38*, 948–952.

Manning, M., Burdon, J., De Silva, N., Meier, X., Pidakala, P., Punter, M., Billing, D. (2016). Maturity and postharvest temperature management affect rot expression in 'Hort16A' kiwifruit. *Postharvest Biology and Technology*, *113*, 40–47.

Maria, T., Tsaniklidis, G., Delis, C., Nikolopoulou, A. E., Nikoloudakis, N., Karapanos, I., Aivalakis, G. (2016). Gene transcript accumulation and enzyme activity of β-amylases suggest involvement in the starch depletion during the ripening of cherry tomatoes. *Plant Gene*, *5*, 8–12.

Marsh, K. B., Friel, E. N., Gunson, A., Lund, C., MacRae, E. (2006). Perception of flavour in standardized fruit pulps with additions of acids or sugars. *Food Quality and Preference*, *17*, 376-386.

Matsumoto, H., Ikoma, Y., Kato, M., Kuniga, T., Nakajima, N., Yoshida, T. (2007). Quantification of carotenoids in citrus fruit by LC-MS and comparison of patterns of seasonal changes for carotenoids among citrus varieties. *Journal of Agricultural and Food Chemistry*, *55*, 2356–2368.

Matsumoto, H., Ikoma, Y., Kato, M., Nakajima, N., Hasegawa, Y. (2009). Effect of postharvest temperature and ethylene on carotenoid accumulation in the flavedo and juice sacs of Satsuma mandarin (*Citrus unshiu* Marc.) fruit. *Journal of Agricultural and Food Chemistry*, *57*, 4724–4732.

Mayuoni, L., Tietel, Z., Patil, B. S., Porat, R. (2011). Does ethylene degreening affect internal quality of citrus fruit?. *Postharvest Biology and Technology*, *62*, 50–58.

McAtee, P. A., Richardson, A. C., Nieuwenhuizen, N. J., Gunaseelan, K., Hoong, L., Chen, X., Atkinson, R. G., Burdon, J. N., David, K. M., Schaffer, R. J. (2015). The hybrid non-ethylene and ethylene ripening response in kiwifruit (*Actinidia chinensis*) is associated with differential regulation of MADS-box transcription factors. *BMC Plant Biology*, *15*, 304. https://doi.org/10.1186/s12870-015-0697-9.

McAtee, P., Karim, S., Schaffer, R. J., David, K. (2013). A dynamic interplay between phytohormones is required for fruit development, maturation, and ripening. *Frontiers in Plant Science*, *4*, 79. https://doi.org/10.3389/fpls.2013.00079.

McCollum, G., Maul, P. (2007). 1-Methylcyclopropene inhibits degreening but stimulates respiration and ethylene biosynthesis in grapefruit. *HortScience*, *42*, 120–124.

McGlasson, W. B., Scott, K. J., Mendoza Jr., D. B. (1979). The refrigerated storage of tropical and subtropical products. *International Journal of Refrigeration*, *2*, 199–206.

McMurchie, E. J., McGlasson, W. B., Eaks, I. L. (1972). Treatment of fruit with propylene gives information about biogenesis of ethylene. *Nature*, *237*, 235–236.

Medina-Puche, L., Cumplido-Laso, G., Amil-Ruiz, F., Hoffmann, T., Ring, L., Rodríguez-Franco, A., Caballero, J. L., Schwab, W., Muñoz-Blanco, J., Blanco-Portales, R. (2013). MYB10 plays a major role in the regulation of flavonoid/phenylpropanoid metabolism during ripening of *Fragaria× ananassa* fruits. *Journal of Experimental Botany*, *65*, 401–417.

Merchante, C., Vallarino, J. G., Osorio, S. *et al*. (2013). Ethylene is involved in strawberry fruit ripening in an organ-specific manner. *Journal of Experimental Botany*, *64*, 4421–4439.

Minas, I. S., Tanou, G., Karagiannis, E., Belghazi, M., Molassiotis, A. (2016). Coupling of physiological and proteomic analysis to understand the ethylene and chilling induced kiwifruit ripening syndrome. *Frontiers in Plant Science*, *7*, 120. https://doi.org/10.3389/fpls.2016.00120.

Mitalo, O. W., Asiche, W. O., Kasahara, Y., Tosa, Y., Owino, W. O., Mworia, E. G., Ushijima, K., Nakano, R., Kubo, Y. (2018b). Characterization of ripening-related genes involved in ethylene-independent low temperature-modulated ripening in 'Rainbow Red' kiwifruit during storage and on-vine. *The Horticulture Journal*, 87, 421–429.

Mitalo, O. W., Asiche, W.O., Kasahara, Y., Tosa, Y., Tokiwa, S., Ushijima, K., Nakano, R., Kubo, Y. (2019a). Comparative analysis of fruit ripening and associated genes in two kiwifruit cultivars ('Sanuki Gold' and 'Hayward') at various storage temperatures. *Postharvest Biology and Technology*, *147*, 20–28.

Mitalo, O. W., Tokiwa, S., Kasahara, Y., *et al*. (2018a). Determination of optimum temperature for long-term storage and analysis of ripening-related genes in 'Rainbow Red' kiwifruit. *Acta Horticulturae*, 1218, 517–524.

Mitalo, O. W., Tokiwa, S., Kondo, Y., *et al*. (2019b). Low temperature storage stimulates fruit softening and sugar accumulation without ethylene and aroma volatile production in kiwifruit. *Frontiers in Plant Science*, *10*, 888. https://doi.org/10.3389/fpls.2019.00888.

Mitalo, O. W., Tosa, Y., Tokiwa, S., *et al*. (2019c). 'Passe Crassane' pear fruit (*Pyrus communis* L.) ripening: Revisiting the role of low temperature via integrated physiological and transcriptome analysis. *Postharvest Biology and Technology*, *158*, 110949. https://doi.org/10.1016/j.postharvbio.2019.110949.

Moscatello, S., Famiani, F., Proietti, S., Farinelli, D., Battistelli, A. (2011). Sucrose synthase dominates carbohydrate metabolism and relative growth rate in growing kiwifruit (*Actinidia deliciosa*, cv Hayward). *Scientia Horticulturae*, *128*, 197–205.

Mou, W., Li, D., Bu, J., Jiang, Y., Khan, Z. U., Luo, Z., Mao, L., Ying, T. (2016). Comprehensive analysis of ABA effects on ethylene biosynthesis and signalling during tomato fruit ripening. *PLoS ONE*, *11*, e0154072. https://doi.org/10.1371/journal.pone.0154072.

Murakami, S., Ikoma, Y., Yano, M. (2014). Low temperature increases ethylene sensitivity in *Actinidia chinensis* 'Rainbow Red' kiwifruit. *Journal of the Japanese Society for Horticultural Science*, *83*, 322–326.

Murakami, S., Ikoma, Y., Yano, M. (2015). Fruit ripening process in red kiwifruit cultivar 'Rainbow Red' (*Actinidia chinensis*) on vines. *Environmental Control in Biology*, *53*, 159–163.

Mworia, E. G., Yoshikawa, T., Salikon, N., Oda, C., Asiche, W. O., Yokotani, N., Abe, D., Ushijima, K., Nakano, R., Kubo, Y. (2012). Low-temperature-modulated fruit ripening is independent of ethylene in 'Sanuki Gold' kiwifruit. *Journal of Experimental Botany*, *63*, 963–971.

Mworia, E. G., Yoshikawa, T., Yokotani, N., Fukuda, T., Suezawa, K., Ushijima, K., Nakano, R., Kubo, Y. (2010). Characterization of ethylene biosynthesis and its regulation during fruit ripening in kiwifruit, *Actinidia chinensis* 'Sanuki Gold'. *Postharvest Biology and Technology*, *55*, 108–113.

Nakano, R., Ogura, E., Kubo, Y., Inaba, A. (2003). Ethylene biosynthesis in detached young persimmon fruit is initiated in calyx and modulated by water loss from the fruit. *Plant Physiology*, *131*, 276–286.

Nakatsuka, A., Murachi, S., Okunishi, H., Shiomi, S., Nakano, R., Kubo, Y., Inaba, A. (1998). Differential expression and internal feedback regulation of 1-aminocyclopropane-1-carboxylate synthase, 1-aminocyclopropane-1-carboxylate oxidase, and ethylene receptor genes in tomato fruit during development and ripening. *Plant Physiology*, *118*, 1295–1305.

Nardozza, S., Boldingh, H. L., Osorio, S., Höhne, M., Wohlers, M., Gleave, A. P., MacRae, E. A., Richardson, A. C., Atkinson, R. G., Sulpice, R., Fernie, A. R., Clearwater, M. J. (2013). Metabolic analysis of kiwifruit (*Actinidia deliciosa*) berries from extreme genotypes reveals hallmarks for fruit starch metabolism. *Journal of Experimental Botany*, *64*, 5049–5063.

Nguyen, T. B. T., Ketsa, S., van Doorn, W. G. (2003). Relationship between browning and the activities of polyphenoloxidase and phenylalanine ammonia lyase in banana peel during low temperature storage. *Postharvest Biology and Technology*, *30*, 187–193.

Nham, N. T., Macnish, A. J., Zakharov, F., Mitcham, E. J. (2017). 'Bartlett' pear fruit (*Pyrus communis* L.) ripening regulation by low temperatures involves genes associated with jasmonic acid, cold response, and transcription factors. *Plant Science*, *260*, 8–18.

Nicolas, P., Lecourieux, D., Kappel, C., Cluzet, S., Cramer, G., Delrot, S., Lecourieux, F. (2014). The basic leucine zipper transcription factor ABSCISIC ACID RESPONSE ELEMENT-BINDING FACTOR2 is an important transcriptional regulator of abscisic acid-dependent grape berry ripening processes. *Plant Physiology*, *164*, 365–383.

Nieuwenhuizen, N. J., Chen, X., Wang, M. Y., Matich, A. J., Perez, R. L., Allan, A. C., Green, S. A., Atkinson, R. G. (2015). Natural variation in monoterpene synthesis in kiwifruit: transcriptional regulation of terpene synthases by NAC and ETHYLENE-INSENSITIVE3-like transcription factors. *Plant Physiology*, *167*, 1243–1258.

Nisar, N., Li, L., Lu, S., Khin, N. C., Pogson, B. J. (2015). Carotenoid metabolism in plants. *Molecular Plant*, *8*, 68–82.

Nishiyama, I., Fukuda, T., Shimohashi, A., Oota, T. (2008). Sugar and organic acid composition in the fruit juice of different *Actinidia* varieties. *Food Science and Technology Research*, *14*, 67–73.

O'Rourke, D. (2011). Dealing with changing world markets for kiwifruit. *Acta Horticulturae*, *913*, 45–50.

Oda-Yamamizo, C., Mitsuda, N., Sakamoto, S., Ogawa, D., Ohme-Takagi, M., Ohmiya, A. (2016). The NAC transcription factor ANAC046 is a positive regulator of chlorophyll degradation and senescence in Arabidopsis leaves. *Scientific Reports*, *6*, 23609. https://doi.org/10.1038/srep23609.

Ohmiya, A., Kato, M., Shimada, T., Nashima, K., Kishimoto, S., Nagata, M. (2019). Molecular Basis of Carotenoid Accumulation in Horticultural Crops. *The Horticulture Journal*, *88*, 135–149.

Ortiz, A., Graell, J., López, M. L., Echeverría, G., Lara, I. (2010). Volatile ester-synthesising capacity in 'Tardibelle' peach fruit in response to controlled atmosphere and 1-MCP treatment. *Food Chemistry*, *123*, 698–704.

Osorio, S., Alba, R., Nikoloski, Z., Kochevenko, A., Fernie, A. R., Giovannoni, J. J. (2012). Integrative comparative analyses of transcript and metabolite profiles from pepper and tomato ripening and development stages uncovers species-specific patterns of network regulatory behaviour. *Plant Physiology*, *159*, 1713–1729.

Osorio, S., Scossa, F., Fernie, A. (2013). Molecular regulation of fruit ripening. *Frontiers in Plant Science*, *4*, 198. https://doi.org/10.3389/fpls.2013.00198.

Park, Y. S., Jung, S. T., Gorinstein, S. (2006). Ethylene treatment of 'Hayward' kiwifruits (*Actinidia deliciosa*) during ripening and its influence on ethylene biosynthesis and antioxidant activity. *Scientia Horticulturae*, *108*, 22−28.

Paul, V., Pandey, R., Srivastava, G. C. (2012). The fading distinctions between classical patterns of ripening in climacteric and non-climacteric fruit and the ubiquity of ethylene-an overview. *Journal of Food Science and Technology*, *49*, 1–21.

Pech, J. C., Bouzayen, M., Latché, A. (2008). Climacteric fruit ripening: ethylene-dependent and independent regulation of ripening pathways in melon fruit. *Plant Science*, *175*, 114–120.

Peng, G., Xie, X. L., Jiang, Q., Song, S., Xu, C. J. (2013). Chlorophyll a/b binding protein plays a key role in natural and ethylene-induced degreening of Ponkan (*Citrus reticulata* Blanco). *Scientia Horticulturae*, *160*, 37–43.

Petroni, K., Tonelli, C. (2011). Recent advances on the regulation of anthocyanin synthesis in reproductive organs. *Plant Science*, *181*, 219–229.

Pirrello, J., Prasad, B. N., Zhang, W., Chen, K., Mila, I., Zouine, M., Latché, A., Pech, J. C., Ohme-Takagi, M., Bouzayen, M. (2012). Functional analysis and binding affinity of tomato ethylene response factors provide insight on the molecular bases of plant differential responses to ethylene. *BMC Plant Biology*, *12*, 190. https://doi.org/10.1186/1471-2229-12-190.

Porat, R. (2008). Degreening of citrus fruit. *Tree and Forestry Science and Biotechnology*, *2*, 71–76.

Pranamornkith, T., East, A., Heyes, J. (2012). Influence of exogenous ethylene during refrigerated storage on storability and quality of *Actinidia chinensis* (cv. 'Hort16A'). *Postharvest Biology and Technology*, *64*, 1−8.

Pratt, H. K., Reid, M. S. (1974). Chinese gooseberry: seasonal patterns in fruit growth and maturation, ripening respiration and the role ethylene. *Journal of the Science of Food and Agriculture*, *25*, 741–757.

Purvis, A. C., Barmore, C. R. (1981). Involvement of ethylene in chlorophyll degradation in peel of citrus fruits. *Plant Physiology*, *68*, 854–856.

Qiu, K., Li, Z., Yang, Z., *et al*. (2015). EIN3 and ORE1 accelerate degreening during ethylene-mediated leaf senescence by directly activating chlorophyll catabolic genes in Arabidopsis. *PLoS Genetics*, *11*, e1005399. https://doi.org/10.1371/journal.pgen.1005399.

Rahim, M. A., Busatto, N., Trainotti, L. (2014). Regulation of anthocyanin biosynthesis in peach fruits. *Planta*, *240*, 913–929.

Ranocha, P., Dima, O., Nagy, R., Felten, J., Corratgé-Faillie, C., Novák, O., Morreel, K., Lacombe, B., Martinez, Y., Pfrunder, S. *et al*. (2013). Arabidopsis WAT1 is a vacuolar auxin transport facilitator required for auxin homoeostasis. *Nature Communications*, *4*, 2625. https://doi.org/10.1038/ncomms3625.

Ravaglia, D., Espley, R. V., Henry-Kirk, R. A., Andreotti, C., Ziosi, V., Hellens, R. P., Costa, G., Allan, A. C. (2013). Transcriptional regulation of flavonoid biosynthesis in nectarine (*Prunus persica*) by a set of R2R3 MYB transcription factors. *BMC Plant Biology*, *13*, 68. https://doi.org/10.1186/1471-2229-13-68.

Redgwell, R. J., Percy, A. E. (1992). Cell wall changes during on–vine softening of kiwifruit. *New Zealand Journal of Crop and Horticultural Science*, *20*, 453–456.

Richardson, A. C., Boldingh, H. L., McAtee, P. A., Gunaseelan, K., Luo, Z., Atkinson, R. G., David, K. M., Burdon, J. N., Schaffer, R. J. (2011). Fruit development of the diploid kiwifruit, *Actinidia chinensis* 'Hort16A'. *BMC Plant Biology*, *11*, 182. https://doi.org/10.1186/1471-2229-11-182.

Ríos, G., Naranjo, M. A., Rodrigo, M. J., Alós, E., Zacarías, L., Cercós, M., Talón, M. (2010). Identification of a GCC transcription factor responding to fruit colour change events in citrus through the transcriptomic analyses of two mutants. *BMC Plant Biology*, *10*, 276. https://doi.org/10.1186/1471-2229-10-276.

Ritenour, M. A., Crisosto, C. H., Garner, D. T., Cheng, G. W., Zoffoli, J. P. (1999). Temperature, length of cold storage and maturity influence the ripening rate of ethylene-preconditioned kiwifruit. *Postharvest Biology and Technology*, *15*, 107–115.

Robinson, M. D., McCarthy, D. J., Smyth, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, *26*, 139–140.

Rodrigo, M. J., Alquézar, B., Alós, E., Lado, J., Zacarías, L. (2013). Biochemical bases and molecular regulation of pigmentation in the peel of citrus fruit. *Scientia Horticulturae*, *163*, 46–62.

Rodrigo, M. J., Alquezar, B., Zacarías, L. (2006). Cloning and characterization of two 9-cis-epoxycarotenoid dioxygenase genes, differentially regulated during fruit maturation and under stress conditions, from orange (*Citrus sinensis* L. Osbeck). *Journal of Experimental Botany*, *57*, 633–643.

Rodrigo, M. J., Marcos, J. F., Alférez, F., Mallent, M. D., Zacarías, L. (2003). Characterization of Pinalate, a novel *Citrus sinensis* mutant with a fruit-specific alteration that results in yellow pigmentation and decreased ABA content. *Journal of Experimental Botany*, *54*, 727–738.

Rodrigo, M. J., Zacarias, L. (2007). Effect of postharvest ethylene treatment on carotenoid accumulation and the expression of carotenoid biosynthetic genes in the flavedo of orange (*Citrus sinensis* L. Osbeck) fruit. *Postharvest Biology and Technology*, *43*, 14–22.

Sagar, M., Chervin, C., Mila, I. *et al*. (2013). SlARF4, an auxin response factor involved in the control of sugar metabolism during tomato fruit development. *Plant Physiology*, *161*, 1362–1374.

Sakuraba, Y., Schelbert, S., Park, S. Y., Han, S. H., Lee, B. D., Andrès, C. B., Kessler, F., Hörtensteiner, S., Paek, N. C. (2012). STAY-GREEN and chlorophyll catabolic enzymes interact at light-harvesting complex II for chlorophyll detoxification during leaf senescence in Arabidopsis. *The Plant Cell*, *24*, 507–518.

Salinero, M. C., Vela, P., Sainz, M. J. (2009). Phenological growth stages of kiwifruit (*Actinidia deliciosa* 'Hayward'). *Scientia Horticulturae*, *121*, 27–31.

Sanchez-Ballesta, M. T., Hommel, M., Jaimes-Miranda, F., Tournier, B., Zegzouti, H., Mila, I., Latché, A., Pech, J. C., Bouzayen, M., Regad, F. (2007). Characterization of tomato Sl-MBF1 transcriptional coactivator gene family. In: Ramina, A., Chang, C., Giovannoni, J., Klee, H., Perata, P., Woltering, E. (Eds.), *Advances in Plant Ethylene Research-Proceedings of the 7th International Symposium on the Plant Hormone Ethylene*. Springer, Dordrecht, pp. 369–375.

Sato, Y., Morita, R., Katsuma, S., Nishimura, M., Tanaka, A., Kusaba, M. (2009). Two short-chain dehydrogenase/reductases, NON-YELLOW COLORING 1 and NYC1-LIKE, are required for chlorophyll b and light-harvesting complex II degradation during senescence in rice. *The Plant Journal*, *57*, 120–131.

Sawamura, M. (1981). Levels of endogenous ethylene in attached citrus fruits. *Agricultural and Biological Chemistry*, *45*, 2935–2937.

Schaart, J. G., Dubos, C., Romero De La Fuente, I., Van Houwelingen, A. M., de Vos, R. C., Jonker, H. H., Xu, W., Routaboul, J. M., Lepiniec, L., Bovy, A. G. (2013). Identification and characterization of MYB-bHLH-WD40 regulatory complexes controlling proanthocyanidin biosynthesis in strawberry (*Fragaria× ananassa*) fruits. *New Phytologist*, *197*, 454–467.

Schaffer, R. J., Friel, E. N., Souleyre, E. J., Bolitho, K., Thodey, K., Ledger, S., Bowen, J. H., Ma, J. H., Nain, B., Cohen, D., Gleave, A. P., Crowhurst, R. N., Janssen, B. J., Yao, J. L., Newcomb, R. D. (2007). A genomics approach reveals that aroma production in apple is controlled by ethylene predominantly at the final step in each biosynthetic pathway. *Plant Physiology*, *144*, 1899–1912.

Schaller, G. E. (2012). Ethylene and the regulation of plant development. *BMC Biology*, *10*, 9. https://doi.org/10.1186/1741-7007-10-9.

Schelbert, S., Aubry, S., Burla, B., Agne, B., Kessler, F., Krupinska, K., Hörtensteiner, S. (2009). Pheophytin pheophorbide hydrolase (pheophytinase) is involved in chlorophyll breakdown during leaf senescence in Arabidopsis. *The Plant Cell*, *21*, 767–785.

Schröder, R., Atkinson, R. G. (2006). Kiwifruit cell walls: towards an understanding of softening?. *New Zealand Journal of Forestry Science*, *36*, 112−129.

Schröder, R., Atkinson, R. G., Langenkämper, G., Redgwell, R. J. (1998). Biochemical and molecular characterization of xyloglucan endotransglycosylase from ripe kiwifruit. *Planta*, *204*, 242–251.

Seymour, G. B., Granell, A. (2014). Fruit development and ripening. *Journal of Experimental Botany*, *65*, 4489–4490.

Seymour, G. B., Ryder, C. D., Cevik, V., Hammond, J. P., Popovich, A., King, G. J., Vrebalov, J., Giovannoni, J. J., Manning, K. (2010). A SEPALLATA gene is involved in the development and ripening of strawberry (*Fragaria× ananassa* Duch.) fruit, a non-climacteric tissue. *Journal of Experimental Botany*, *62*, 1179–1188.

Sfakiotakis, E., Antunes, M. D., Stavroulakis, G., Niklis, N., Ververidis, P., Gerasopoulos, D. (1997). Ethylene biosynthesis and its regulation in ripening "Hayward" kiwifruit. In *Biology and Biotechnology of the Plant Hormone Ethylene* (pp. 47–56). Springer, Dordrecht.

Shan, W., Kuang, J. F., Chen, L., Xie, H., Peng, H. H., Xiao, Y. Y., Li, X. P., Chen, W. X., He, Q. G., Chen, J. Y., Lu, W. J. (2012). Molecular characterization of banana NAC transcription factors and their interactions with ethylene signalling component EIL during fruit ripening. *Journal of Experimental Botany*, *63*, 5171–5187.

Shemer, T. A., Harpaz-Saad, S., Belausov, E., Lovat, N., Krokhin, O., Spicer, V., Standing, K. G., Goldschmidt, E. E., Eyal, Y. (2008). Citrus chlorophyllase dynamics at ethylene-induced fruit color-break: a study of chlorophyllase expression, posttranslational processing kinetics, and in situ intracellular localization. *Plant Physiology*, *148*, 108–118.

Shimoda, Y., Ito, H., Tanaka, A. (2016). Arabidopsis STAY-GREEN, Mendel's green cotyledon gene, encodes magnesium-dechelatase. *The Plant Cell*, *28*, 2147–2160.

Sisler, E. C., Serek, M. (1997). Inhibitors of ethylene responses in plants at the receptor level: recent developments. *Physiologia Plantarum*, *100*, 577–582.

Sisler, E. C., Yang, S. F. (1984). Ethylene, the gaseous plant hormone. *Bioscience*, *34*, 234–238.

Sitrit, Y., Bennett, A. B. (1998). Regulation of tomato fruit polygalacturonase mRNA accumulation by ethylene: a re-examination. *Plant Physiology*, *116*, 1145–1150.

Slavin, J. L., Lloyd, B. (2012). Health benefits of fruits and vegetables. *Advances in nutrition*, *3*, 506–516.

Snelgar, W. P., Hopkirk, G., McPherson, H. G. (1993). Predicting harvest date for kiwifruit: variation of soluble solids concentration with mean temperature. *New Zealand Journal of Crop and Horticultural Science*, *21*, 317–324.

Sobhy, I. S., Miyake, A., Shinya, T., Galis, I. (2017). Oral secretions affect HIPVs induced by generalist (*Mythimna loreyi*) and specialist (*Parnara guttata*) herbivores in rice. *Journal of Chemical Ecology*, *43*, 929–943.

Song, C. B., Shan, W., Yang, Y. Y., Tan, X. L., Fan, Z. Q., Chen, J. Y., Lu, W. J., Kuang, J. F. (2018). Heterodimerization of MaTCP proteins modulates the transcription of *MaXTH10/11* genes during banana fruit ripening. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*, *1861*, 613–622.

Song, Y., Yang, C., Gao, S., Zhang, W., Li, L., Kuai, B. (2014). Age-triggered and dark-induced leaf senescence require the bHLH transcription factors PIF3, 4, and 5. *Molecular Plant*, *7*, 1776–1787.
Souleyre, E. J., Greenwood, D. R., Friel, E. N., Karunairetnam, S., Newcomb, R. D. (2005). An alcohol acyl transferase from apple (cv. Royal Gala), MpAAT1, produces esters involved in apple fruit flavor. *The FEBS Journal*, *272*, 3132–3144.
Su, L., Diretto, G., Purgatto, E., Danoun, S., Zouine, M., Li, Z., Roustan, J. P., Bouzayen, M., Giuliano, G., Chervin, C. (2015). Carotenoid accumulation during tomato fruit ripening is modulated by the auxin-ethylene balance. *BMC Plant Biology*, *15*, 114. https://doi.org/10.1186/s12870-015-0495-4.
Sun, H., Fan, H. J., Ling, H. Q. (2015). Genome-wide identification and characterization of the *bHLH* gene family in tomato. *BMC Genomics*, *16*, 9. https://doi.org/10.1186/s12864-014-1209-2.
Sun, L., Sun, Y., Zhang, M. *et al*. (2012). Suppression of 9-cis-epoxycarotenoid dioxygenase, which encodes a key enzyme in abscisic acid biosynthesis, alters fruit texture in transgenic tomato. *Plant Physiology*, *158*, 283–298.
Suo, J., Li, H., Ban, Q., Han, Y., Meng, K., Jin, M., Zhang, Z., Rao, J. (2018). Characteristics of chilling injury-induced lignification in kiwifruit with different sensitivities to low temperatures. *Postharvest Biology and Technology*, *135*, 8–18.
Suzuki, N., Bajad, S., Shuman, J., Shulaev, V., Mittler, R. (2008). The transcriptional co-activator MBF1c is a key regulator of thermotolerance in *Arabidopsis thaliana*. *Journal of Biological Chemistry*, *283*, 9269–9275.
Tacken, E., Ireland, H., Gunaseelan, K. *et al*. (2010). The role of ethylene and cold temperature in the regulation of the apple POLYGALACTURONASE1 gene and fruit softening. *Plant Physiology*, *153*, 294–305.
Takos, A. M., Jaffé, F. W., Jacob, S. R., Bogs, J., Robinson, S. P., Walker, A. R. (2006). Light-induced expression of a MYB gene regulates anthocyanin biosynthesis in red apples. *Plant Physiology*, *142*, 1216–1232.
Tani, E., Tsaballa, A., Stedel, C. *et al*. (2011). The study of a SPATULA-like bHLH transcription factor expressed during peach (*Prunus persica*) fruit development. *Plant Physiology and Biochemistry*, *49*, 654–663.
Tao, N., Wang, C., Xu, J., Cheng, Y. (2012). Carotenoid accumulation in postharvest "Cara Cara" navel orange (*Citrus sinensis* Osbeck) fruits stored at different temperatures was transcriptionally regulated in a tissue-dependent manner. *Plant Cell Reports*, *31*, 1667–1676.
Tatsuki, M., Nakajima, N., Fujii, H., Shimada, T., Nakano, M., Hayashi, K., Hayama, H., Yoshioka, H., Nakamura, Y. (2013). Increased levels of IAA are required for system 2 ethylene synthesis causing fruit softening in peach (*Prunus persica* L. Batsch). *Journal of Experimental Botany*, *64*, 1049–1059.
Thomashow, M. F. (2010). Molecular basis of plant cold acclimation: insights gained from studying the CBF cold response pathway. *Plant Physiology*, *154*, 571–577.
Tian, M. S., Prakash, S., Zhang, N., Ross, G. S. (2002). Chilling-induced ethylene biosynthesis in Braeburn apples. *Plant Growth Regulation*, *38*, 249–257.
Tieman, D. M., Taylor, M. G., Ciardi, J. A., Klee, H. J. (2000). The tomato ethylene receptors NR and LeETR4 are negative regulators of ethylene response and exhibit functional compensation within a multigene family. *Proceedings of the National Academy of Science*, *97*, 5663–5668.
Trainotti, L., Tadiello, A., Casadoro, G. (2007). The involvement of auxin in the ripening of climacteric fruits comes of age: the hormone plays a role of its own and has an intense interplay with ethylene in ripening peaches. *Journal of Experimental Botany*, *58*, 3299–3308.
Tucker, G., Yin, X., Zhang, A., Wang, M., Zhu, Q., Liu, X., Xie, X., Chen, K., Grierson, D. (2017). Ethylene and fruit softening. *Food Quality and Safety*, *1*, 253–267.
Van Wyk, A. A., Huysamer, M., Barry, G. H. (2009). Extended low-temperature shipping adversely affects rind colour of 'Palmer Navel' sweet orange [*Citrus sinensis* (L.) Osb.] due to carotenoid degradation but can partially be mitigated by optimising post-shipping holding temperature. *Postharvest Biology and Technology*, *53*, 109–116.
Vicente, A. R., Saladie, M., Rose, J. K., Labavitch, J. M. (2007). The linkage between cell wall metabolism and fruit softening: looking to the future. *Journal of the Science of Food and Agriculture*, *87*, 1435–1448.
Vico, I., Jurick, W. M., Camp, M. J., Janisiewicz, W. J., Conway, W. S. (2011). Temperature suppresses decay on apple fruit by affecting *Penicillium solitum* conidial germination, mycelial growth and polygalacturonase activity. *Plant Pathology Journal*, *9*, 144–148.
Villalobos-Acuña, M., Mitcham, E. J. (2008). Ripening of European pears: The chilling dilemma. *Postharvest Biology and Technology*, *49*, 187–200.
Vrebalov, J., Ruezinsky, D., Padmanabhan, V., White, R., Medrano, D., Drake, R., Wolfgang, S., Giovannoni, J. (2002). A MADS-box gene necessary for fruit ripening at the tomato ripening-inhibitor (rin) locus. *Science*, *296*, 343–346.
Wan, C. Y., Wilkins, T. A. (1994). A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). *Analytical Biochemistry*, *223*, 7–12.
Wang, A., Tan, D., Takahashi, A., Zhong Li, T., Harada, T. (2007). MdERFs, two ethylene-response factors involved in apple fruit ripening. *Journal of Experimental Botany*, *58*, 3743–3748.
Wang, C. Y. (1993). Approaches to reduce chilling injury of fruits and vegetables. *Horticultural Reviews*, *15*, 63–95.
Wang, H., Ma, F., Cheng, L. (2010). Metabolism of organic acids, nitrogen and amino acids in chlorotic leaves of 'Honeycrisp' apple (*Malus domestica* Borkh) with excessive accumulation of carbohydrates. *Planta*, 232, 511–522.

Wang, K. L. C., Li, H., Ecker, J. R. (2002). Ethylene biosynthesis and signalling networks. *The Plant Cell*, *14*, S131–S151.
Wang, K., Yin, X. R., Zhang, B., Grierson, D., Xu, C. J., Chen, K. S. (2017). Transcriptomic and metabolic analyses provide new insights into chilling injury in peach fruit. *Plant, Cell and Environment*, *40*, 1531–1551.
Wang, P., Zhang, B., Li, X., Xu, C., Yin, X., Shan, L., Ferguson, I., Chen, K. (2009). Ethylene signal transduction elements involved in chilling injury in non-climacteric loquat fruit. *Journal of Experimental Botany*, *61*, 179–190.
Wang, X., Yin, W., Wu, J., Chai, L., Yi, H. (2016). Effects of exogenous abscisic acid on the expression of citrus fruit ripening-related genes and fruit ripening. *Scientia Horticulturae*, *201*, 175–183.
Wang, Z. Y., MacRae, E. A., Wright, M. A., Bolitho, K. M., Ross, G. S., Atkinson, R. G. (2000). Polygalacturonase gene expression in kiwifruit: relationship to fruit softening and ethylene production. *Plant Molecular Biology*, *42*, 317–328.
Wasternack, C., Hause, B. (2013). Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. *Annals of Botany*, *111*, 1021–1058.
Watkins, C. B. (2006). The use of 1-methylcyclopropene (1-MCP) on fruits and vegetables. *Biotechnology Advances*, *24*, 389–409.
Wellburn, A. R. (1994). The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *Journal of Plant Physiology*, *144*, 307–313.
Weng, L., Zhao, F., Li, R., Xu, C., Chen, K., Xiao, H. (2015). The zinc finger transcription factor SlZFP2 negatively regulates abscisic acid biosynthesis and fruit ripening in tomato. *Plant Physiology*, *167*, 931–949.
Whittaker, D. J., Smith, G. S., Gardner, R. C. (1997). Expression of ethylene biosynthetic genes in *Actinidia chinensis* fruit. *Plant Molecular Biology*, 34, 45–55.
Wills, R., Golding, J. (2016). *Postharvest: An introduction to the physiology and handling of fruit and vegetables* (6th ed). Wallingford, Oxfordshire, UK: CABI Publishing, pp. 1−16.
Wu, G. A., Prochnik, S., Jenkins, J., *et al*. (2014). Sequencing of diverse mandarin, pummelo and orange genomes reveals complex history of admixture during citrus domestication. *Nature Biotechnology*, *32*, 656–662.
Xiao, Y. Y., Kuang, J. F., Qi, X. N., Ye, Y. J., Wu, Z. X., Chen, J. Y., Lu, W. J. (2018). A comprehensive investigation of starch degradation process and identification of a transcriptional activator MabHLH6 during banana fruit ripening. *Plant Biotechnology Journal*, *16*, 151–164.
Xie, X. B., Li, S., Zhang, R. F., Zhao, J., Chen, Y. C., Zhao, Q., Yao, Y. X., You, C. X., Zhang, X. S., Hao, Y. J. (2012). The bHLH transcription factor MdbHLH3 promotes anthocyanin accumulation and fruit colouration in response to low temperature in apples. *Plant, Cell and Environment*, *35*, 1884–1897.
Xu, F., Yuan, S., Zhang, D. W., Lv, X., Lin, H. H. (2012). The role of alternative oxidase in tomato fruit ripening and its regulatory interaction with ethylene. *Journal of Experimental Botany*, *63*, 5705–5716.
Yamaguchi, S. (2008). Gibberellin metabolism and its regulation. *Annual Review of Plant Biology*, *59*, 225–251.
Yang, S., Xu, C., Zhang, B., Li, X., Chen, K. (2007). Involvement of both subgroups A and B of *Expansin* genes in kiwifruit fruit ripening. *HortScience*, 42, 315–319.
Yang, Y. Y., Shan, W., Kuang, J. F., Chen, J. Y., Lu, W. J. (2019). Four HD-ZIPs are involved in banana fruit ripening by activating the transcription of ethylene biosynthetic and cell wall-modifying genes. *Plant Cell Reports*, 1–12.
Yano, M., Hasegawa, Y. (1993). Ethylene production in harvested kiwifruit with special reference to ripe rot. *Journal of the Japanese Society for Horticultural Science*, *62*, 443–449.
Yin, X. R., Allan, A. C., Chen, K. S., Ferguson, I. B. (2010). Kiwifruit EIL and ERF genes involved in regulating fruit ripening. *Plant Physiology*, *153*, 1280–1292.
Yin, X. R., Allan, A. C., Xu, C. J., Burdon, J., Dejnoprat, S., Chen, K. S., Ferguson, I. B. (2012). Differential expression of kiwifruit ERF genes in response to postharvest abiotic stress. *Postharvest Biology and Technology*, *66*, 1–7.
Yin, X. R., Allan, A. C., Zhang, B., Wu, R. M., Burdon, J., Wang, P., Ferguson, I. B., Chen, K. S. (2009). Ethylene-related genes show a differential response to low temperature during 'Hayward' kiwifruit ripening. *Postharvest Biology and Technology*, *52*, 9–15.
Yin, X. R., Chen, K. S., Allan, A. C., Wu, R. M., Zhang, B., Lallu, N., and Ferguson, I. B. (2008). Ethylene-induced modulation of genes associated with the ethylene signalling pathway in ripening kiwifruit. *Journal of Experimental Botany*, *59*, 2097–2108.
Yin, X. R., Xie, X. L., Xia, X. J., Yu, J. Q., Ferguson, I. B., Giovannoni, J. J., Chen, K. S. (2016). Involvement of an ethylene response factor in chlorophyll degradation during citrus fruit degreening. *The Plant Journal*, *86*, 403–412.
Yip, W. K., Moore, T., Yang, S. F. (1992). Differential accumulation of transcripts for four tomato 1-aminocyclopropane-1-carboxylate synthase homologs under various conditions. *Proceedings of the National Academy of Sciences*, *89*, 2475–2479.
Yokotani, N., Nakano, R., Imanishi, S., Nagata, M., Inaba, A., Kubo, Y. (2009). Ripening-associated ethylene biosynthesis in tomato fruit is autocatalytically and developmentally regulated. *Journal of Experimental Botany*, *60*, 3433–3442.
Yuan, H., Zhang, J., Nageswaran, D., Li, L. (2015). Carotenoid metabolism and regulation in horticultural crops. *Horticulture Research*, *2*, 15036. https://doi.org/10.1038/hortres.2015.36.

Yun, Z., Jin, S., Ding, Y., Wang, Z., Gao, H., Pan, Z., Xu, J., Chen, Y., Deng, X. (2012). Comparative transcriptomics and proteomics analysis of citrus fruit, to improve understanding of the effect of low temperature on maintaining fruit quality during lengthy post-harvest storage. *Journal of Experimental Botany*, *63*, 2873–2893.

Zeng, J. K., Li, X., Xu, Q., Chen, J. Y., Yin, X. R., Ferguson, I. B., Chen, K. S. (2015). EjAP2-1, an AP2/ERF gene, is a novel regulator of fruit lignification induced by chilling injury, via interaction with EjMYB transcription factors. *Plant Biotechnology Journal*, *13*, 1325–1334.

Zhang, B., Horvath, S. (2005). A general framework for weighted gene co-expression network analysis. *Statistical Applications in Genetics and Molecular Biology*, *4*, 17. https://doi.org/10.2202/1544-6115.1128.

Zhang, B., Liu, J., Yang, Z. E., Chen, E. Y., Zhang, C. J., Zhang, X. Y., Li, F. G. (2018). Genome-wide analysis of GRAS transcription factor gene family in *Gossypium hirsutum* L. *BMC Genomics*, *19*, 348. https://doi.org/10.1186/s12864-018-4722-x.

Zhang, B., Tieman, D. M., Jiao, C., Xu, Y., Chen, K., Fei, Z., Giovannoni, J. J., Klee, H. J. (2016). Chilling-induced tomato flavor loss is associated with altered volatile synthesis and transient changes in DNA methylation. *Proceedings of the National Academy of Sciences*, *113*, 12580–12585.

Zhang, B., Yin, X. R., Li, X., Yang, S. L., Ferguson, I. B., Chen, K. S. (2009). Lipoxygenase gene expression in ripening kiwifruit in relation to ethylene and aroma production. *Journal of Agricultural and Food Chemistry*, *57*, 2875–2881.

Zhang, M. J., Jiang, Y. M., Jiang, W. B., Liu, X. J. (2006). Regulation of ethylene synthesis of harvested banana fruit by 1-methylcyclopropene. *Food Technology and Biotechnology*, *44*, 111–115.

Zhang, M., Yuan, B., Leng, P. (2009). The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit. *Journal of Experimental Botany*, *60*, 1579–1588.

Zhang, Y. J., Wang, X. J., Wu, J. X., Chen, S. Y., Chen, H., Chai, L. J., Yi, H. L. (2014). Comparative transcriptome analyses between a spontaneous late-ripening sweet orange mutant and its wild type suggest the functions of ABA, sucrose and JA during citrus fruit ripening. *PLoS One*, *9*, e116056. https://doi.org/10.1371/journal.pone.0116056.

Zhang, Y., Bouwmeester, H. J., Kappers, I. F. (2019). Combined transcriptome and metabolome analysis identifies defence responses in spider mite-infested pepper (*Capsicum annuum*). *Journal of Experimental Botany*, *71*, 330–343.

Zhang, Y., Liu, Z., Chen, Y., He, J. X., Bi, Y. (2015). PHYTOCHROME-INTERACTING FACTOR 5 (PIF5) positively regulates dark-induced senescence and chlorophyll degradation in Arabidopsis. *Plant Science*, *237*, 57–68.

Zhang, Z., Smart, T. J., Choi, H., Hardy, F., Lohans, C. T., Abboud, M. I., Richardson, M. S. W., Paton, R. S., McDonough, M. A., Schofield, C. J. (2017). Structural and stereoelectronic insights into oxygenase-catalysed formation of ethylene from 2-oxoglutarate. *Proceedings of the National Academy of Sciences*, *114*, 4667–4672.

Zhang, Z., Zhang, H., Quan, R., Wang, X. C., Huang, R. (2009). Transcriptional regulation of the ethylene response factor LeERF2 in the expression of ethylene biosynthesis genes controls ethylene production in tomato and tobacco. *Plant Physiology*, *150*, 365–377.

Zhu, A., Li, W., Ye, J., Sun, X., Ding, Y., Cheng, Y., Deng, X. (2011). Microarray expression profiling of postharvest Ponkan mandarin (*Citrus reticulata*) fruit under cold storage reveals regulatory gene candidates and implications on soluble sugars metabolism. *Journal of Integrative Plant Biology*, *53*, 358–374.

Zhu, M., Chen, G., Zhou, S., Tu, Y., Wang, Y., Dong, T., Hu, Z. (2013). A new tomato NAC (NAM/ATAF1/2/CUC2) transcription factor, SlNAC4, functions as a positive regulator of fruit ripening and carotenoid accumulation. *Plant and Cell Physiology*, *55*, 119–135.