Running title: Low temperature promotes peel degreening in citrus fruit

Low temperature transcriptionally modulates natural peel degreening in lemon (*Citrus limon* L.) fruit independently of endogenous ethylene

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Citrus peel degreening is promoted by low temperature via modulation of multiple genes associated with chlorophyll degradation, carotenoid biosynthesis, photosystem disassembly, phytohormones and transcription factors without involving ethylene signalling.

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 25ºC, moderately low temperatures (5ºC, 10ºC, 15ºC and 20ºC) 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.

Keywords: 1-methylcyclopropene, Carotenoids, Chlorophyll, Citrus, Ethylene, Low temperature, Peel degreening, Phytohormones, Transcriptome

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*., 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 13ºC 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 (6–15ºC) 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 (0ºC, 5ºC, 10ºC, 15ºC, 20ºC, and 25ºC). We found that both ethylene treatment and moderately low temperatures (0ºC, 5ºC, 10ºC, 15ºC and 20ºC; 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.

Materials and methods

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 25ºC 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 5ºC, 10ºC, 15ºC, 20ºC or 25ºC for up to 42 d. Additionally, three separate sets (40 fruit each) were stored at either 5ºC, 15ºC or 25ºC with repeated (twice a week) 12 h 1-MCP treatments. Fruit peel (flavedo) was sampled, frozen in liquid nitrogen and stored at -80ºC for future analysis, each sample containing three biological replicates.

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.

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 20ºC 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.

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 Table S1, while the multiple-reaction-monitoring mode of the tandem quadrupole mass spectrometer and precursor-product ion transitions for each compound are listed in Table S2.

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 5ºC, 15ºC and 25ºC. 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 by 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, 5ºC vs. 25ºC and/or 15ºC vs. 25ºC. 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.

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 5ºC, 10ºC, 15ºC, 20ºC and 25ºC. 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 (Table S3) 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). *AcActin* (*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.

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.

Results

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. 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.

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. 1B, peel colour of fruit at 5ºC, 10ºC, 15ºC and 20ºC 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 15ºC followed by 10ºC and 20ºC, than 5ºC at which fruit retained appreciable greenish colour even after 42 d. In contrast, fruit at 25ºC 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 5ºC and 15ºC but not at 25ºC, notwithstanding the repeated 1-MCP treatments (Fig. 1C). Together, these findings suggested that low temperature may promote peel colour changes in lemon fruit independently of ethylene.

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 5ºC and 15ºC were examined against those at 25ºC.

RNA-seq analysis resulted in the identification of 3105 DEGs (q-value < 0.001), which responded to either ethylene or low temperature (Fig. 2). Ethylene had the largest share, influencing 2329 DEGs as opposed to 5ºC and 15ºC that influenced 1634 and 597 DEGs, respectively (Fig. 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, 5ºC and/or 15ºC (Fig. 2B). Ethylene treatment exclusively upregulated and downregulated 592 and 700 genes, respectively (Fig. 2C, D). Likewise, an aggregate of 337 and 439 genes were exclusively upregulated and downregulated, respectively by 5ºC and 15ºC. The remaining DEGs (420 upregulated and 617 downregulated) were jointly influenced by either ethylene, 5ºC and/or 15ºC. Detailed information about the DEGs showing specific and shared responses to ethylene, 5ºC and/or 15ºC is listed in Tables S4–S10. 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.

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. 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. 1A). During storage, peel chlorophyll content also decreased in fruit at moderately low temperatures (5ºC, 10ºC, 15ºC and 20ºC), whereas they were maintained at high levels in fruit at 25ºC. It is noteworthy that peel chlorophyll content also decreased in fruit at 5ºC and 15ºC despite repeated treatments with 1-MCP.

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. 3B, Table S11). 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. 3C). It is however worth noting that repeated 1-MCP treatments did not suppress the increased expression of *ClPPH* at 5ºC and 15ºC.

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. S1), 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. 4A). However, while 1-MCP treatment significantly inhibited carotenoid changes induced by ethylene treatment, repeated 1-MCP treatments did not abolish peel carotenoid decrease at 5ºC and 15ºC.

By examining the RNA-seq data, we identified 13 DEGs that had been associated with carotenoid metabolism (Fig. 4B, Table S11). 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. 4C). Additionally, the expression of all the three analysed genes increased in the peel of fruit at 5ºC and 15ºC despite the repeated 1-MCP treatments.

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. 5A, Table S11). 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. 5B). Nevertheless, repeated 1-MCP treatments did not suppress the expression decrease induced at 5ºC and 15ºC, 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. 6A). Most of the ethylene-related genes were up-regulated by ethylene treatment, while low temperature, especially 5ºC, 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 5ºC. 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 up-regulated both after 4 d of ethylene exposure, and 28 d of storage at lower temperatures (5ºC, 10ºC, 15ºC and 20ºC) than 25ºC (Fig. 6B). There was also a significant increase in *ClNCED5* expression in fruit that were repeatedly treated with 1-MCP at 5ºC and 15ºC. 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. 6C). 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. 7A, Table S11). 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. 7B). In contrast, *ClERF3* was exclusively upregulated by low temperature since marginal expression levels were registered in ethylene-treated fruit (Fig. 7C). Finally, *ClbHLH25* expression increased both upon ethylene treatment and after storage at lower temperatures than 25ºC (Fig. 7C). It is also noteworthy that repeated 1-MCP treatments failed to abolish the upregulation of *ClERF3* and *ClbHLH25* at 5ºC and 15ºC (Fig. 7B, C).

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. 8A, 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 22.5ºC on 3rd September to 3.7ºC 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 13ºC. Interestingly, CCI increased rapidly from -14.2 to -1.1

between 12th October and 13th December when the minimum temperatures were maintained at below 13ºC. 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. 8B). Equally, peel degreening was also accompanied by a gradual decline in the peel content of lutein, β-carotene and α-carotene (Fig. 8C).

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. 8D), both of which were earlier shown to be influenced by low temperature (Fig. 3C, 5B). 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. 8E), which were earlier shown to be upregulated by low temperature (Fig. 4C). 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 13ºC (Fig. 8F). Altogether, these observations demonstrated strong similarities between on-tree and low temperature-modulated peel degreening, as well as their dissimilarities with ethylene-induced changes.

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. 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. 1B). Because of the known involvement of ethylene in citrus degreening (Fig. 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. 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. 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. 2). Ethylene-specific genes such as *ClCLH1* and *ClERF114* were not differentially expressed during low temperature storage (Fig. 3C, 7B), 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. 4C, 7C). 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*, *ClLHCB2*, *ClPSY1*, *ClLCYb2a*, *ClNCED5* and *ClbHLH25* were differentially regulated by either ethylene or low temperature (Fig. 3C, 4C, 5B, 6B, 7D), 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. 3A), which could be attributed to the upregulation of *ClCLH1* and *ClPPH* (Fig. 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).

The peel carotenoid content decreased upon degreening in response to both ethylene and low temperature (Fig. 4A). 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. 1) could be attributed to the small but significant levels of lutein, β-carotene and α-carotene (Fig. 4A), 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. 4B; 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. 5) 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

499 decreased during ethylene-induced and natural peel degreening in 'Ponkan' mandarins.
500 However, the results of the present study suggest that the decrease in the expression of
501 photosystem-encoding genes during natural peel degreening could be stimulated by low
502 temperature independently of ethylene.
503
504 Besides ethylene, various phytohormones such as ABA, GA and JA have been implicated in
505 the peel colour changes that occur during citrus fruit maturation. Peel degreening was shown
506 to be accompanied by an increase in ABA content (Goldschmidt *et al*., 1973), as well as in
507 the expression of ABA biosynthetic and signalling elements (Rodrigo *et al*., 2006; Kato *et al*.,
508 2006). In addition, exogenous ABA accelerated fruit ripening and enhanced fruit colour
509 development (Wang *et al*., 2016), while ABA-deficient citrus mutants showed a delay in the
510 rate of peel degreening (Rodrigo *et al*., 2003). In this study, ABA levels increased in
511 ethylene-treated and low temperature-stored fruit (Fig. 6C), accompanied by an increase in
512 the expression of ABA biosynthetic and signalling genes (Fig. 6A, B). These findings,
513 together with previous reports, suggest that ABA has a positive regulatory role in either
514 ethylene-induced or low temperature-modulated peel degreening in lemon fruit. GA, on the
515 other hand, is known to retard peel colour change. GA application on green citrus fruit was
516 shown to cause a significant delay in peel colour break (Alós *et al*., 2006; Rodrigo and
517 Zacarias, 2007; Rios *et al*., 2010). It is therefore logical that the transcript levels of the GA
518 biosynthetic gene (*ClGA20ox2*) would decrease, whereas those of GA degradatory genes
519 (*ClGA2ox4* and *GA2ox8*) would increase during peel degreening caused by either ethylene or
520 low temperature (Fig. 6A). JAs have thus far been studied in the context of plant adaptive
521 responses to various biotic and abiotic stresses (Zhang *et al*., 2019). However, JA has also
522 been shown to promote fruit ripening in citrus (Zhang *et al*., 2014), strawberry (Concha *et al*.,
523 2013) and tomato (Liu *et al*., 2012). This is consistent with the present findings as lemon fruit
524 degreening was accompanied by an increase in the levels of JA-Ile (Fig. 6C), which is the
525 active conjugate of JA. Additionally, the expression of a large number of JA biosynthetic and
526 signalling-related genes were independently upregulated by either ethylene treatment or low
527 temperature (Fig. 6A).
528
529 Developmentally regulated plant processes such as peel degreening are typically influenced
530 by TFs. Various TFs such as AtNAC046, AtPIF4, AtPIF5, AtORE1 and AtEIN3 were shown
531 to significantly enhance leaf senescence in Arabidopsis by promoting the activity of
532 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. 7). Therefore, ethylene-induced degreening is most likely to be regulated by ethylene-specific TFs such as *ClERF114* (Fig. 7B) and shared TFs such as *ClbHLH25* (Fig. 7D). In contrast, low temperature-modulated degreening could be regulated by specific TFs such as *ClERF3* (Fig. 7C), as well as shared ones such as *ClbHLH25* (Fig. 7D).

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 13ºC (Fig. 8A–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. 3C, 7B) did not show any significant changes in expression during on-tree peel degreening (Fig. 8D, 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. 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. 7D–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 5ºC, yet the peel degreening rate was significantly slower than in fruit at 10ºC, 15ºC and 20ºC. This is probably due to low activity of peel degreening-associated enzymes at 5ºC, 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.

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.

Supplementary data

601 Fig. S1: Chromatograms showing the identified carotenoids.
602 Table S1: Liquid chromatography conditions for phytohormone analysis.
603 Table S2: Parameters for LC-ESI-MS/MS analysis of phytohormones.
604 Table S3: Primer sequences used for RT-qPCR.
605 Table S4: DEGs exclusively responding to ethylene.
606 Table S5: DEGs responding to ethylene and 5ºC.
607 Table S6: DEGs responding to ethylene and 15ºC.
608 Table S7: DEGs responding to ethylene, 5ºC and 15ºC.
609 Table S8: DEGs exclusively influenced by 5ºC.
610 Table S9: DEGs influenced only by 5ºC and 15ºC.
611 Table S10: DEGs exclusively influenced by 15ºC.
612 Table S11: Selected DEGs associated with peel degreening.
613

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Figure legends

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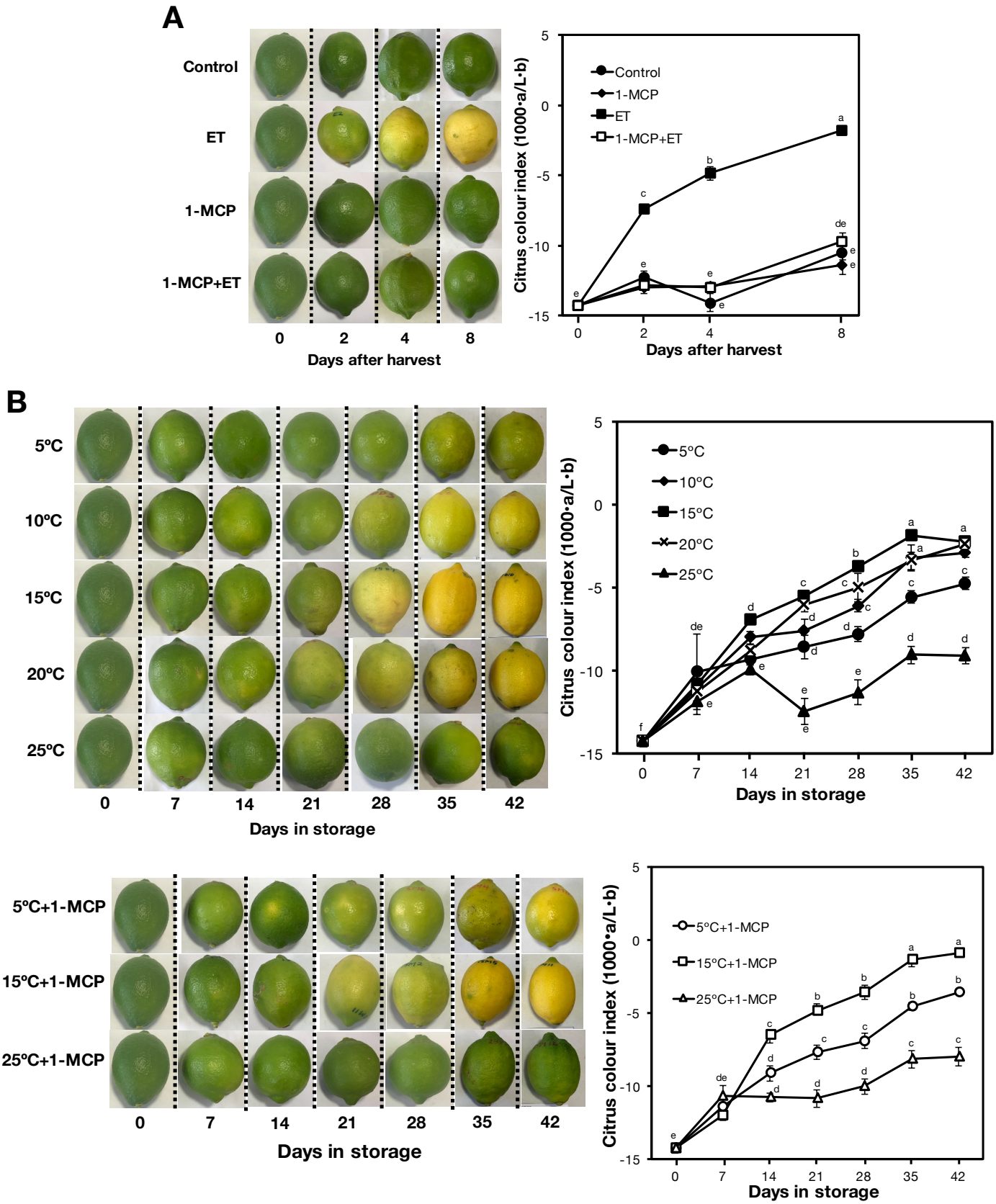

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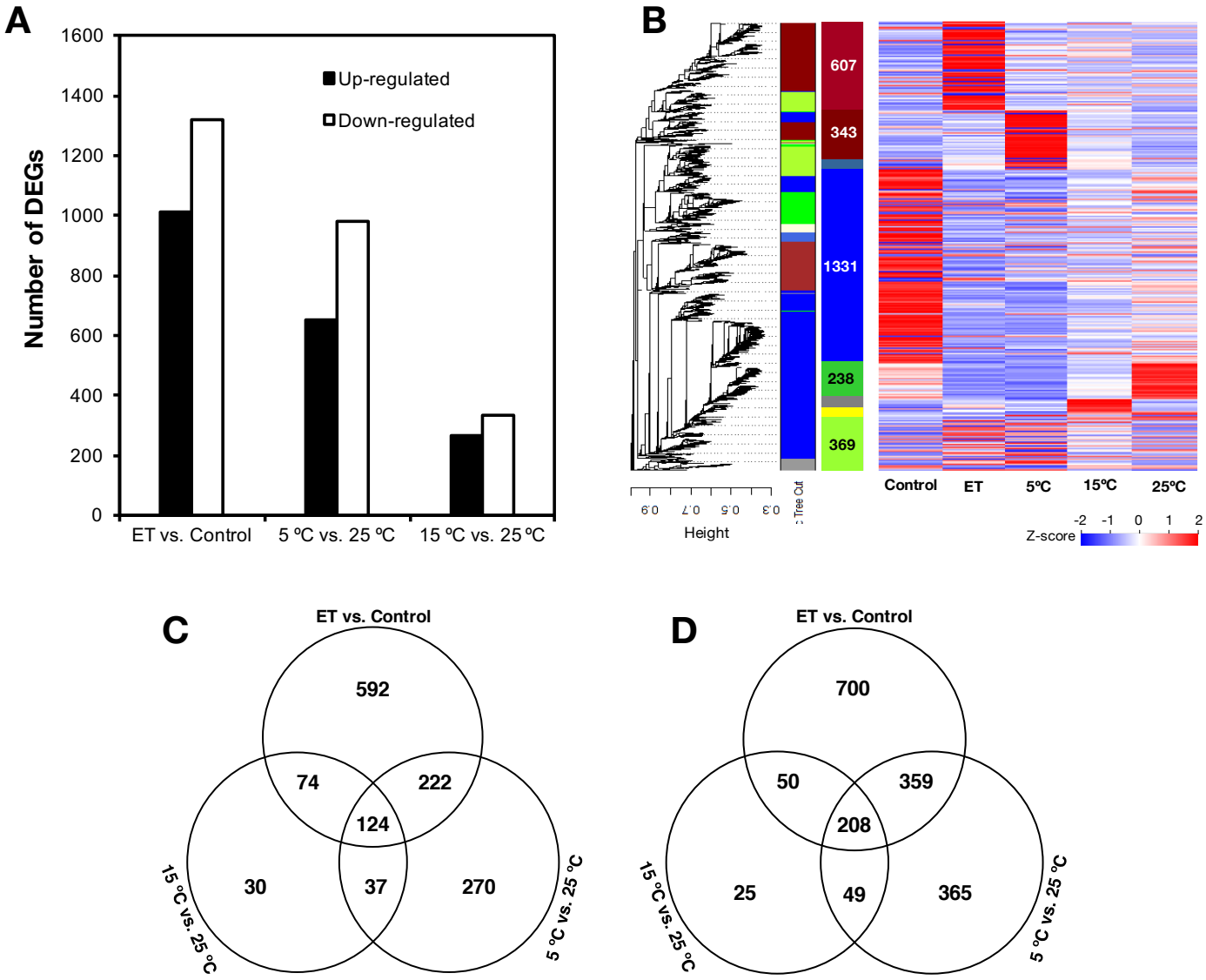

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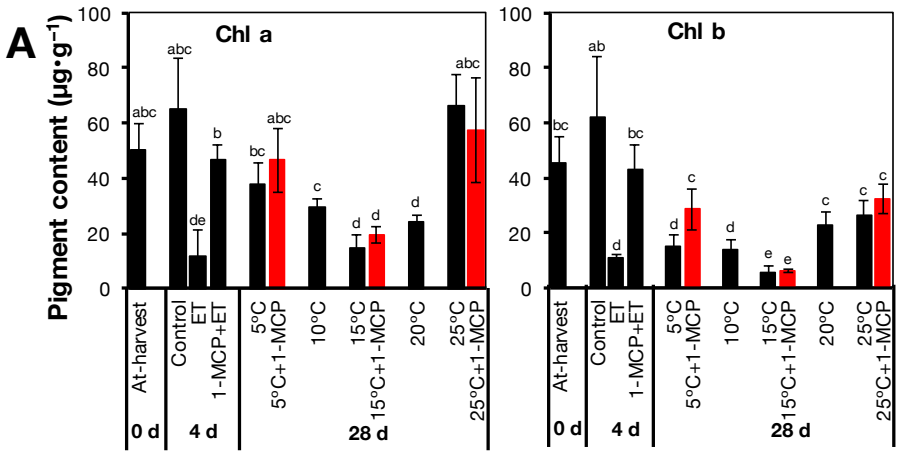

B

Transcript	Symbol	Annotation	ET	5ºC	15ºC
Ciclev10021103m.g	*CLH1*	*Chlorophyllase 1*			
Ciclev10021095m.g	*CLH2*	*Chlorophyllase 2*			
Ciclev10005453m.g	*CLH3*	*Chlorophyllase 3*			
Ciclev10031731m.g	*PPH*	*Pheophytinase*			
Ciclev10004154m.g	*CHLH*	*Magnesium-chelatase subunit chlH*			
Ciclev10014840m.g	*PaO1*	*Pheophorbide a oxygenase 1*			
Ciclev10014836m.g	*PaO2*	*Pheophorbide a oxygenase 2*			
Ciclev10026041m.g	*ACD2*	*Red chlorophyll catabolite reductase*			
Ciclev10008520m.g	*PORA*	*Protochlorophyllide reductase*			
Ciclev10008936m.g	*GGR*	*Geranylgeranyl reductase*			

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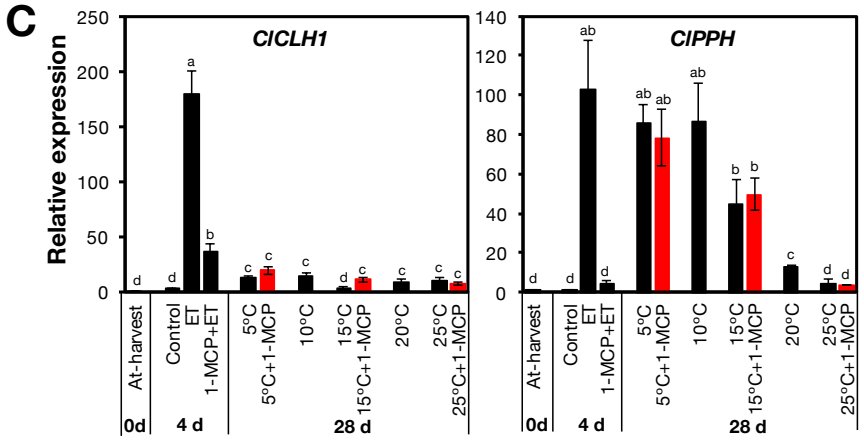

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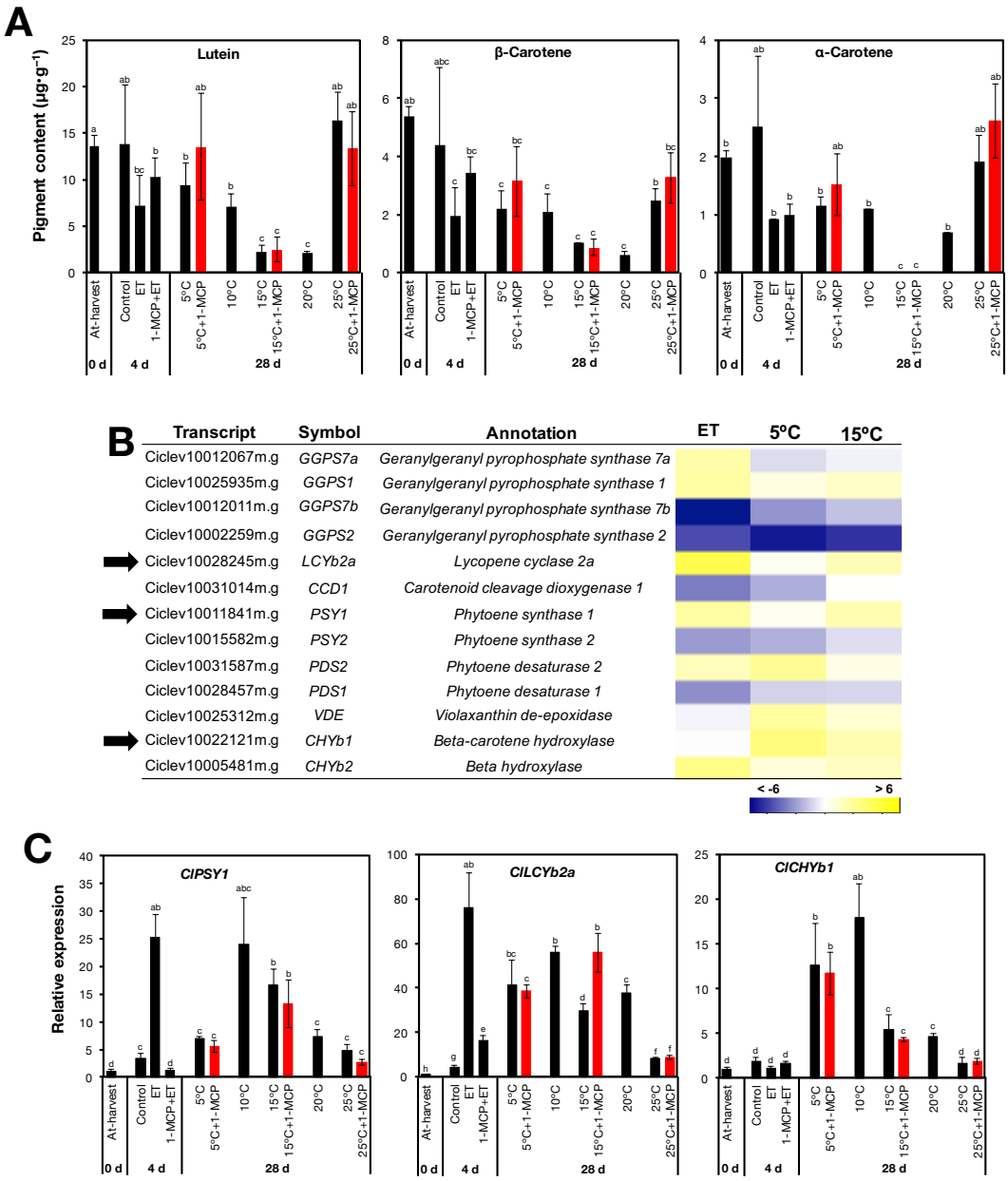

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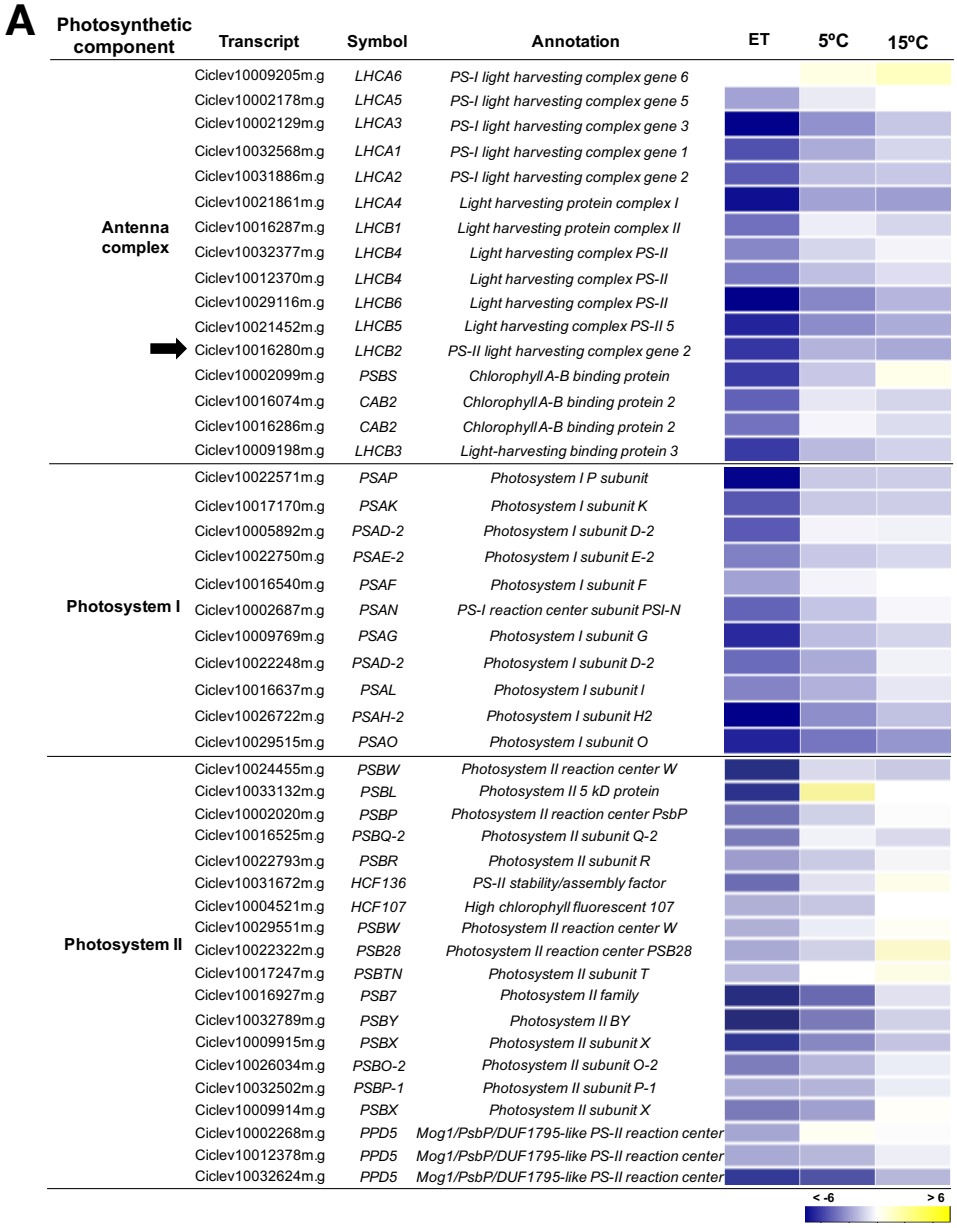

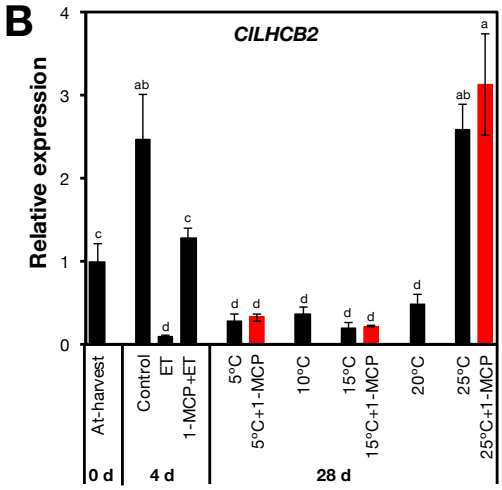

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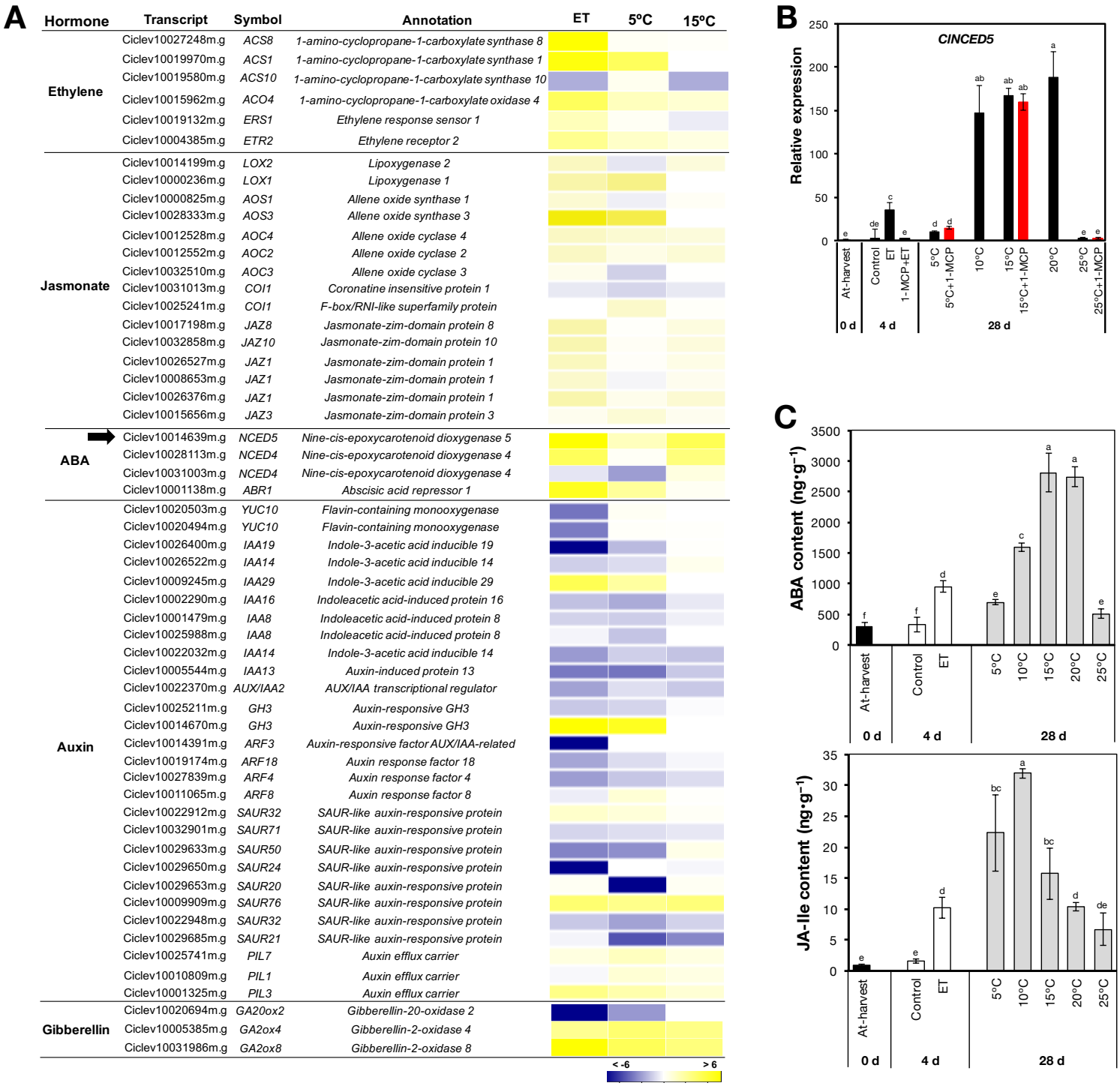

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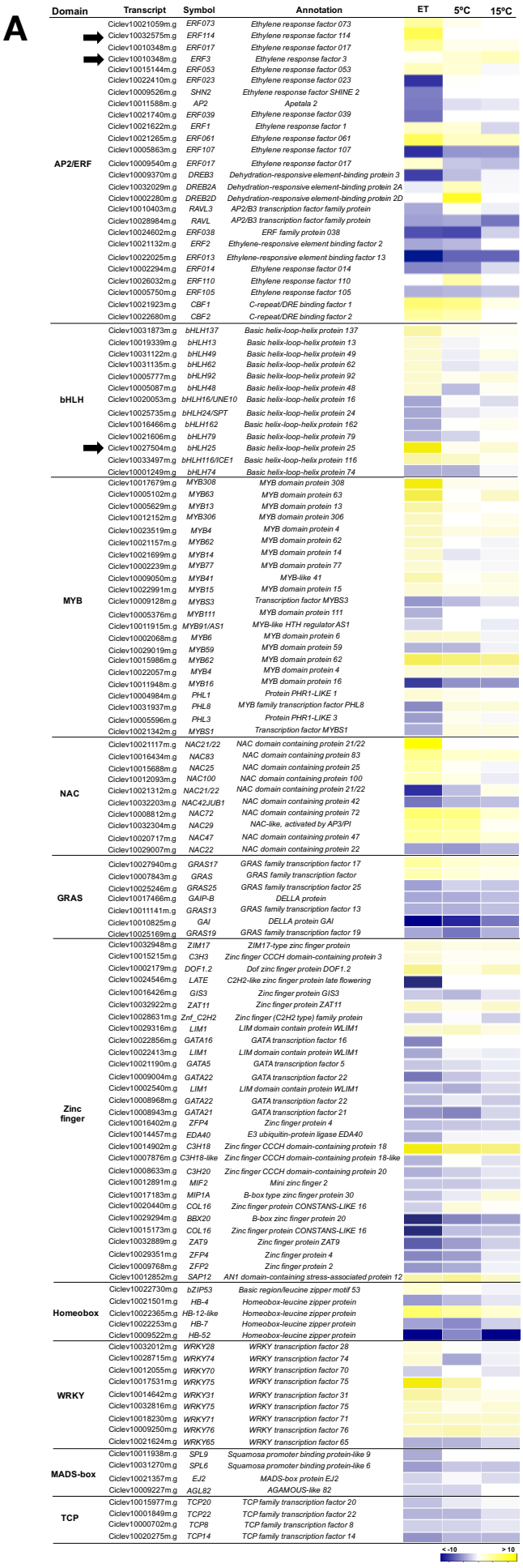

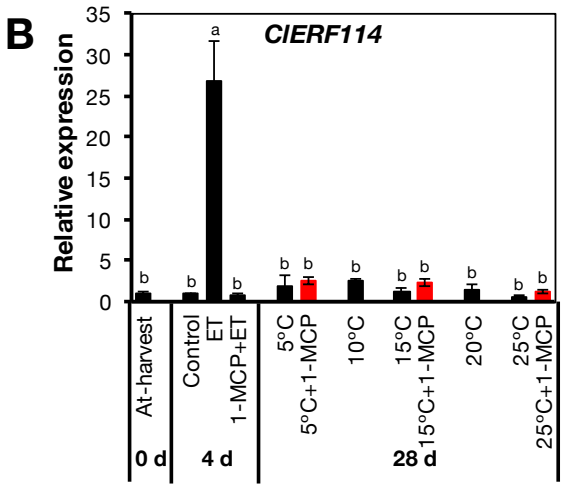

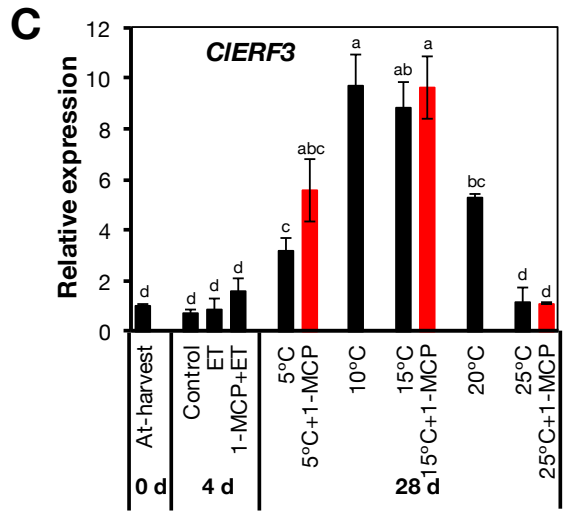

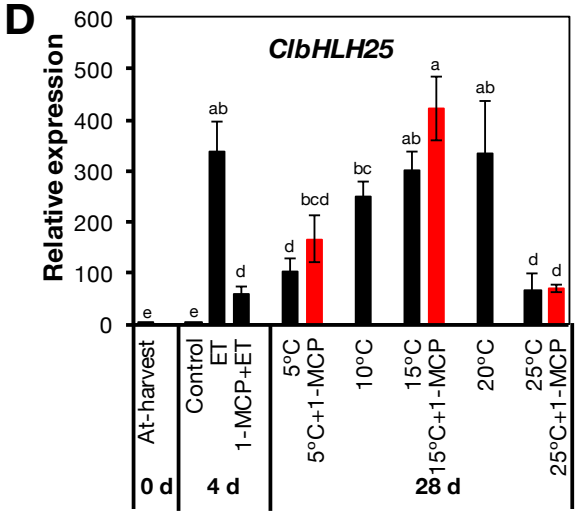

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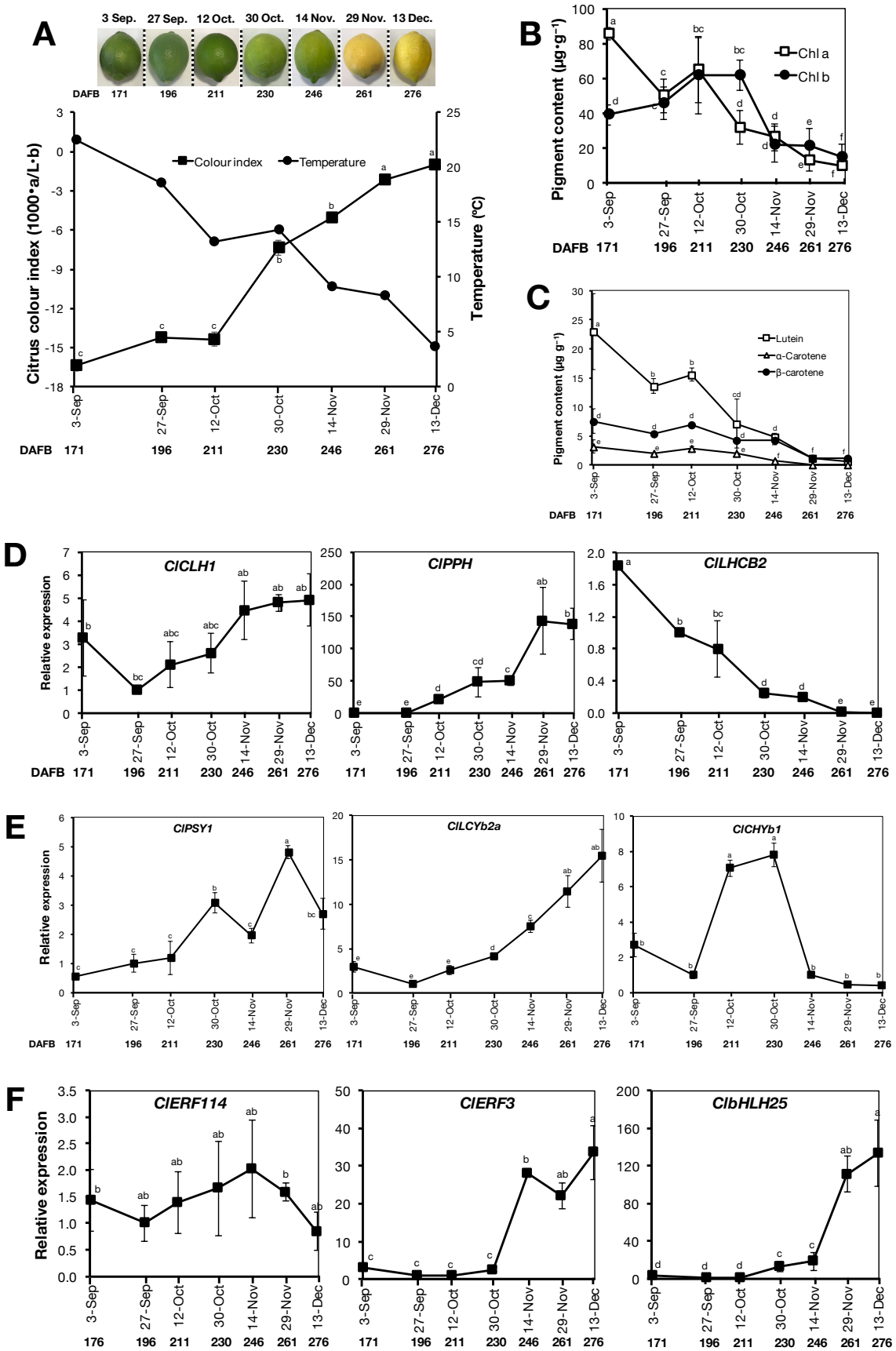

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