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Induction of ethylene in avocado fruit in response to chilling stress on tree

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Summary

Chilling of avocado fruit (*Persea americana* cv. Arad) in the orchard caused a dramatic induction of fruit ripening and a parallel increase in ethylene biosynthesis and receptor genes' expression during shelf life. In-orchard chilling stress stimulated ethylene and CO_2 production already in fruit attached to the tree, and these reduced thereafter during 20 °C storage. In non-chilled control fruit, ethylene and CO_2 production started after 3 d at 20 °C and exhibited a climacteric peak. In-orchard chilling stress also led to membrane destruction expressed as higher electrical conductivity (EC) in chilling stressed (CS) fruit and accelerated softening compared with control fruit. The increase in ethylene production on the day of harvest in CS fruit was accompanied by high expression of two 1-aminocyclopropane-1-carboxylic aCSd (ACC) synthase genes: *PaACS1* and *PaACS2*, and ACC oxidase *PaACO*. The initial gene expressions of *PaACS1*, *PaACS2*, and *PaACO* in the CS fruit at the day of harvest was similar to the levels reached by the control fruit after 4 d at 20 °C. The expression levels of both *PaETR* and *PaERS1* in CS fruit on tree were 25 times higher than the control. In control fruit, expression of ethylene receptor genes was very low at harvest and increased in parallel to the onset of the climacteric ethylene peak. *PaCTR1* transcript levels were less affected by chilling stress, and small changes (less than 3-fold) were observed in CS fruit on the day of harvest. Together, our results suggest that ethylene biosynthesis and ethylene response-pathway genes are involved in regulation of ethylene responsiveness in response to in-orchard chilling stress and during ripening.
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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; CS, chilling stressed; *PaACO*, ACC oxidase; *PaACS*, *Persea americana* ACC synthase; *PaCTR1*, constitutive triple response; *PaERS1*, ethylene response sensor; *PaETR*, ethylene receptor.

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Introduction

Avocado fruit (*Persea americana* Mill.) is a subtropical climacteric fruit, which unlike other fruits does not produce ethylene and does not ripen while attached to the tree (Biale, 1941; Bower and Cutting, 1988). Abeles coined the term ''tree factor'' to describe a putative inhibitor of ethylene production in attached fleshy fruit (Abeles, 1973). Subtropical fruits, including avocado, are sensitive to low temperature, and chilling injury occurs when the plants are exposed to temperatures below 7 °C (Abeles et al., 1992). Cold nights before harvesting stimulated ethylene production in ''Cox Orange Pippin'' apple (Streif, 1976). Bartlett pear from cooler preharvest temperature at different locations exhibited higher ethylene production (Agar et al., 1999). Postharvest cold temperature (−1 °C, low-temperature conditioning) stimulates ethylene biosynthesis during subsequent ripening at room temperature in pears fruit (Knee, 1987). It has been reported that in-orchard chilling conditions increase the rates of ethylene production in avocado fruit 1 d after harvest, showing physiological changes of avocado fruit in connection with chilling stress (Fuchs et al., 1975). It was recently noted that low temperature adversely affects plant growth by inhibition of water and nutrient uptake, changes in membrane fluidity and protein and nucleic acid conformation (Chinnusamy et al., 2007). In avocado, cold storage and ethylene application reduced membrane integrity, expressed as higher electrical conductivity (EC) levels, leading to injury and mesocarp discoloration (Hershkovitz et al., 2005, 2009).

The ethylene biosynthesis pathway is well established in higher plants and exhibits two-step regulatory control. The first step, catalyzed by the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS), involves the formation of ACC from *S*-adenosyl-L-methionine. The second step, catalyzed by ACC oxidase (ACO), converts this intermediate to ethylene. The expression of ACC synthase genes is deferentially and tightly regulated by various developmental, environmental, and hormonal signals (Kende, 1993). Trace amounts of ACC and very low ACS activity were found in preclimacteric avocado fruit. This was followed by a marked increase during the climacteric rise, reaching a peak shortly before the ethylene peak (Sitrit et al., 1986). ACO activity, on the other hand, increased markedly only at the upsurge of ripening ethylene (Owino et al., 2002). It was shown that cold storage stimulated ACO and ACS gene expression in apples (Tian et al., 2002) and in pears (El-Sharkawy et al., 2004; Fonseca et al., 2005).

The ethylene hormone is perceived by a family of integral membrane receptors, which act as negative regulators of the ethylene response pathway (Hua and Meyerowitz, 1998; Tieman et al., 2000). The study of ethylene response-pathway elements and their expression patterns has become an important aspect of fruit ripening studies in various fruit species (Owino et al., 2002; Wiersma et al., 2007).

The present study was carried out to investigate the changes occurring in softening, membrane integrity, respiration, ethylene production, and in genes involved in ethylene biosynthesis, perception and the signal transduction pathway in avocado fruit in response to in-orchard chilling stress.

Material and methods

Plant material

Avocado fruit plants (*Persea americana* Mill. cv. Arad) were obtained from the orchard of Kibbutz Maabarot in the central coastal region of Israel. Fruits were harvested from two different plots in the orchard with low and regular night temperatures. Chilling stressed (CS) fruits were harvested from a plot located at a lower level in which low night temperatures (−3 to −6 °C) occurred for six consecutive nights in January 2008, 2 weeks before harvest. The non-chilled control fruits were harvested from a plot located in an elevated plot of the orchard with regular night temperatures (8–12 °C).

Ethylene and CO_2 production

Individual fruits (4 fruits/treatment) were sealed in 2 L glass jars and held at 20 °C for 1 h. Headspace gas samples were then withdrawn using a 10 mL syringe from each jar. Ethylene content and carbon dioxide in the gas sample were determined with a gas chromatograph (GC) as described by Pesis et al. (1994). The same measurements were repeated every day over 3–5 d at 20 °C. Day 0 represents moment of the harvest.

Fruit firmness and electrical conductivity (EC)

Fruit firmness (N) was determined on whole, unpeeled fruit using an electronic penetrometer (LTCM, Chatillon, New York, NY, USA) with a 6.5-mm conical probe. Penetration for 12 mm was performed at two equidistant points on the equatorial regions of each fruit with a speed of 3 mm s^{-1}.

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The EC was measured on the two opposite sides of the equatorial region of each fruit, including the peel, as described previously (Hershkovitz et al., 2005).

RNA extraction and isolation of cDNA fragments

Samples designated as zero were collected immediately after harvest in the orchard and placed in liquid nitrogen. Total RNA was extracted from the frozen mesocarp tissue using phenol-SDS as described by Or et al. (2000). The first strand cDNAs were synthesized by reverse transcriptase (ABgene, Epson, UK) from 1 μg of total RNA, pre-treated with 1.5 units of RQ1 (Promega, Madison, WI, USA). The ethylene receptor *PaERS1* cDNA fragment was amplified using the specific primers designed against *PaERS1* (Owino et al., 2002) and the *PaCTR1* cDNA fragments of the genes were amplified using the degenerate primers designed on the basis of conserved sequence motifs of the *CTR* genes. The primers were as follows: *PaERS1* (F) 5′-GAGCAAAACGCTGCCTTAGAT-3′, (R)5′-ATTCCTAA-GACCAACAGCCC-3′, *PaCTR1* (F)5′-GGKGCWGGGTC-WTTTGGKACWGT-3′, and (R)5′-CAAATCACGTGGAA-TCTCAAG-3′. Reactions were carried out by a Mastercycler gradient (Eppendorf, Hamburg, Germany) using the following thermocycling profile: 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 56 °C for 1.5 min, 1 min for 72 °C, and a final extension for 7 min at 72 °C. The PCR products were subcloned into a pGEM-T Easy vector System I (Promega, Madison, WI, USA) and were sequenced using both SP6 and T7 primers (Hy-labs Laboratory, Rehovot, Israel). Sequence data were deposited in the GenBank database for *PaCTR1* cDNA under accession number EU417962.

Expression analysis by quantitative real-time PCR (qRT-PCR)

Accumulation of *PaACS1* (AF500119), *PaACS2* (AF500120), *PaACO* (M32692.1), *PaETR* (EU370699), *PaERS1* (AF500121), and *PaCTR1* (EU417962), was evaluated by qRT-PCR with Absolute QPCR SYBR Green ROX Mix (ABgene, Epson, UK). Reactions were carried out using 5 μL of SYBR Green PCR Master mix, 200 nM of each primer in a Rotor GENE 6000 instrument (Corbett Life Science, Sydney, Australia). Specific primers were as reported in Table 1. PCR reactions were performed under the following conditions: 95 °C for 15 min, and then 40 cycles and 1 min at 72 °C. Each cycle included denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s and extension at 72 °C for 20 s. The amount of specific transcript was calculated following the comparative C_T method (Livak and Schmittgen, 2001).

Results and discussion

Ethylene and CO_2 production

The high level of ethylene production in CS fruit reached its maximum value of 80 μL kg^{-1} h^{-1} immediately at the moment of harvest (time 0), indicating that ripening processes had begun already while fruits were attached to the tree (Figure 1A). CS fruit had a significantly higher respiration rate than control fruit (Figure 1B). These data are in agreement of those of Fuchs et al., who showed in other avocado cultivars, the same phenomena of high ethylene and CO_2 production 1 d after harvest in chilling exposed avocado fruit (Fuchs et al., 1975). In contrast, no ethylene production was detected in control fruit on the day of harvest (Figure 1A), supporting the previous finding that avocado in normal growth regimes fails to ripen and to produce ethylene while attached to the tree (Biale, 1941; Bower and Cutting, 1988). The typical climacteric peak of ethylene appeared on day 4 after harvest and reached a maximum value of 120 μL kg^{-1} h^{-1} (Figure 1A). This suggests that putative inhibition of ethylene production in attached fruit may be replaced entirely or in part

Table 1. Specific primers used for transcription analysis by qRT-PCR mRNA.

Primer reverse (5′–3′)	Primer forward (5′–3′)	Gene
TCACCGTACGCTCCTCGTTT	TGATCGGCATACCATCTTGTACA	*PaETR*
CCCGACAACCAGAATCCTTA	ATGGCATGTGTTGAAAAGCA	*PaERS1*
AATGCGGCATCCAAACATTG	TGGCACCAGCTCTATGCAAA	*PaCTR1*
TCCGAAACTCGACATCTTTCG	GCCTCTCAAAAGATCTGGGCT	*PaACS1*
TTGGGACGAGGAATGCATC	GTTCAACCCGGATCGAATAGTTA	*PaACS2*
TTTCCACGGCCTTCATCTTC	CCGAAATTCGTATTCGAGG	*PaACO*
TTCCTTTAAGTTTCAGCCTTG	GTTACTTTAGGACTCCGCC	*r18S*
ACCTGCTGTCACCCACCAAGT	CAAAGCTGCAATCAAGGAGGA	*PaGAPDH*

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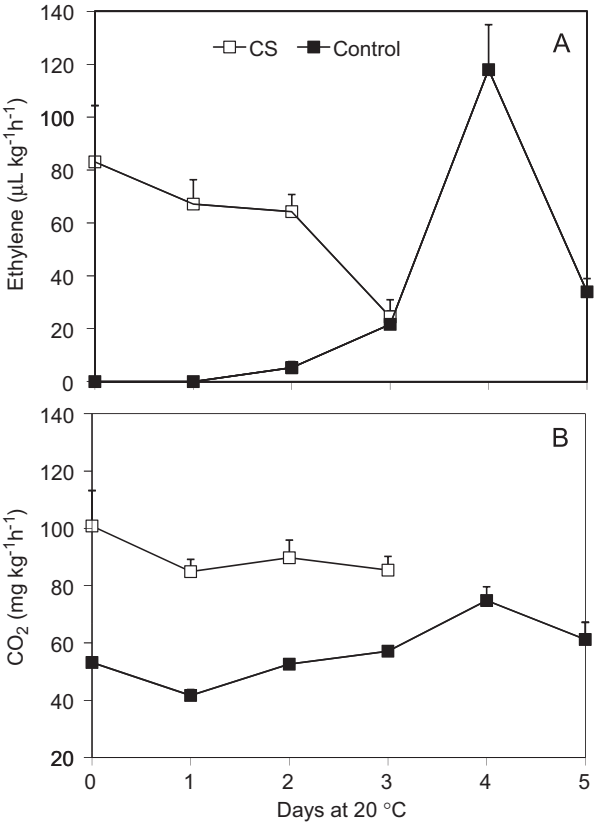

Figure 1. Ethylene (A) and CO_2 (B) production in avocado cv. Arad chilling stressed (CS) as compared with control fruit after harvest during 3–5 d of storage at 20 °C. Vertical bars represent SD of four samples.

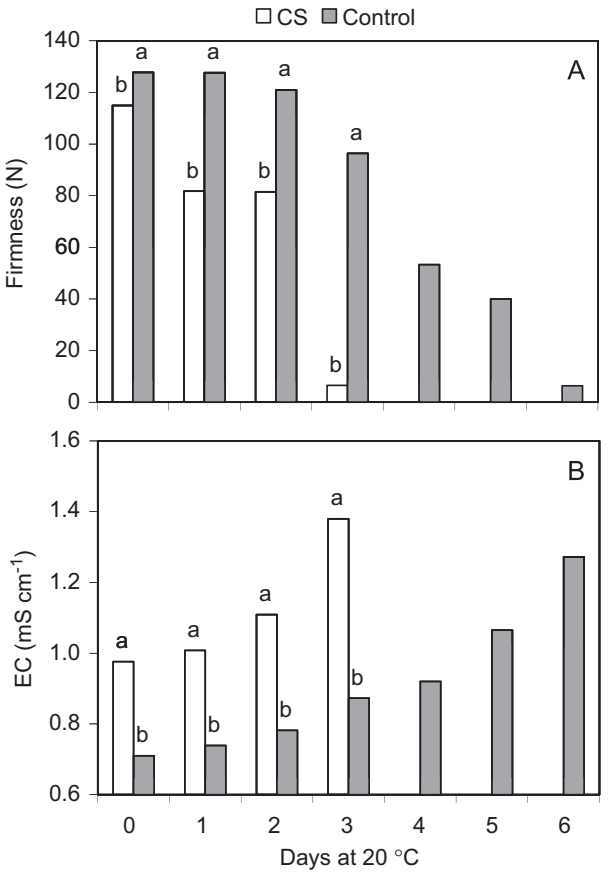

Figure 2. Firmness (A) and electrical conductivity (B) in avocado chilling stressed (CS) as compared with control fruit after harvest during 3–5 d of storage at 20 °C. Columns with a different letter within each treatment are significantly different ($P<0.05$, $n = 16$).

by chilling stress in the orchard, likely via a stimulation of ethylene biosynthesis. Chilling did have an effect on ethylene production and led to a reduction in the time to reach the onset of autocatalytic ethylene rise at 20 °C in Braeburn apple (Tian et al., 2002). Exposure of pears to low temperature (−1 °C) also promotes ethylene synthesis and carbon dioxide production in a number of cultivars (Knee, 1987). Moreover, cold requirements for the development of ripening capacity in pear were dependent on growing season and location, and pear fruits with cooler preharvest temperatures had higher ethylene production rates during ripening (Agar et al., 1999).

Fruit firmness and electrical conductivity

Changes in avocado firmness and EC are illustrated in Figure 2. The softening of CS fruits was significantly accelerated compared with their control counterparts. On the day of harvest, the firmness value of CS fruit was 117 N compared with 129 N in control (Figure 2A), indicating that ripening processes in CS fruit had started. CS fruits completed their ripening, and firmness values fell to 6 N after 3 d at 20 °C, whereas control fruits became soft after 6 d (Figure 2A).

Values of the derived EC in the equatorial region showed marked differences between CS and control fruit. The EC level in CS fruit at time 0 was significantly higher than in control, 1.0 vs. 0.7 mS cm^{-1} (Figure 2B), respectively. The first step in the plant response to low temperature is change in membrane rigidity (Chinnusamy et al., 2007). In avocado fruit, it has been shown that the EC serves as a good indicator of membrane permeability, and that it is highly correlated with ethylene production and with softening (Montoja et al., 1994). The increase in EC in CS fruit already at time 0 verifies our previous observation showing that cold storage enhanced the process of membrane destruction in avocado fruit (Hershkovitz et al., 2005) and the induction of higher EC levels by ethylene treatment (Hershkovitz et al., 2009).

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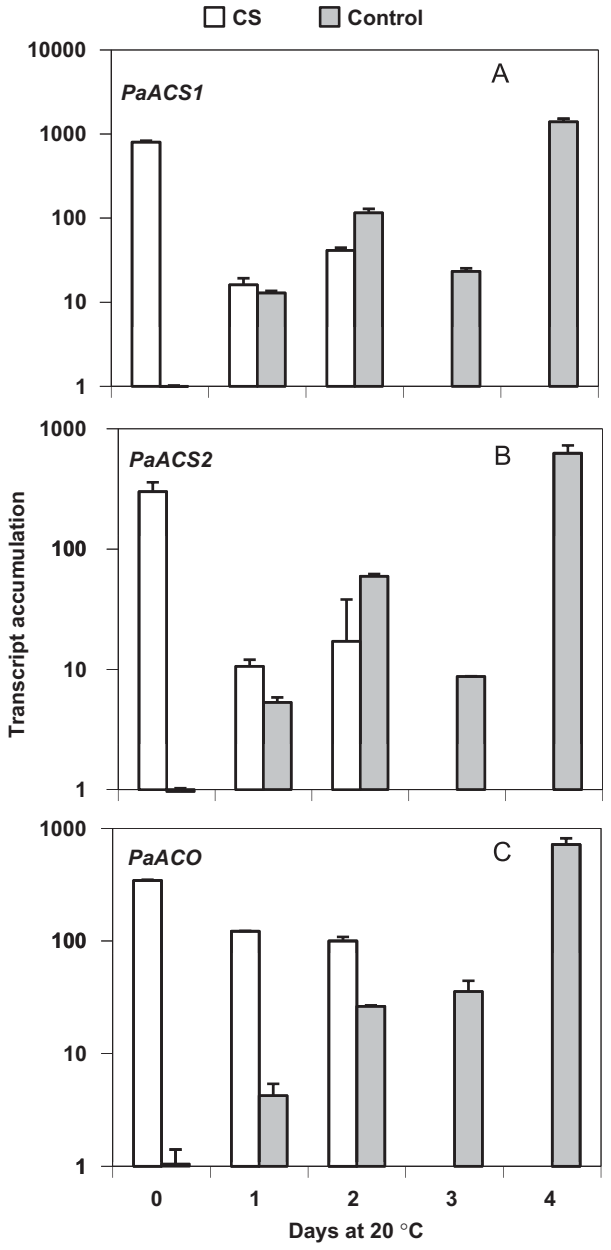

Figure 3. Transcript accumulation of avocado ACC synthase *PaACS1* (A), *PaACS2* (B), and ACC oxidase *PaACO* (C) in chilling stressed (CS) and control fruit after harvest during 2–4 d of storage at 20 °C. Values have been normalized to control on day 0, arbitrarily set to 1. Vertical bars represent SD.

Expression of genes encoding enzymes involved in ethylene biosynthesis

In-orchard chilling stress significantly induced transcript accumulation of *PaACS1*, *PaACS2*, and *PaACO* on the day of harvest, which showed a 100–1000-fold increase in expression compared with controls (Figure 3A–C). Chilling-induced accumulation of both *ACS* and *ACO* transcripts was found in some cultivars of pears and apples after cold storage (Knee, 1987; Tian et al., 2002; El-Sharkawy et al., 2004; Fonseca et al., 2005). In contrast, *PaACS1*, *PaACS2*, and *PaACO* transcript levels in control non-chilled avocado were very low on the day of harvest and increased markedly during ripening, reaching high levels after 4 d at 20 °C, similar to those of CS fruits at time 0 (Figure 3A–C). Thus, the current study verifies previous observations, which have shown detectable expression of avocado *ACS* and *ACO* genes and very low activity of ACS and ACO at harvest that increased in correlation

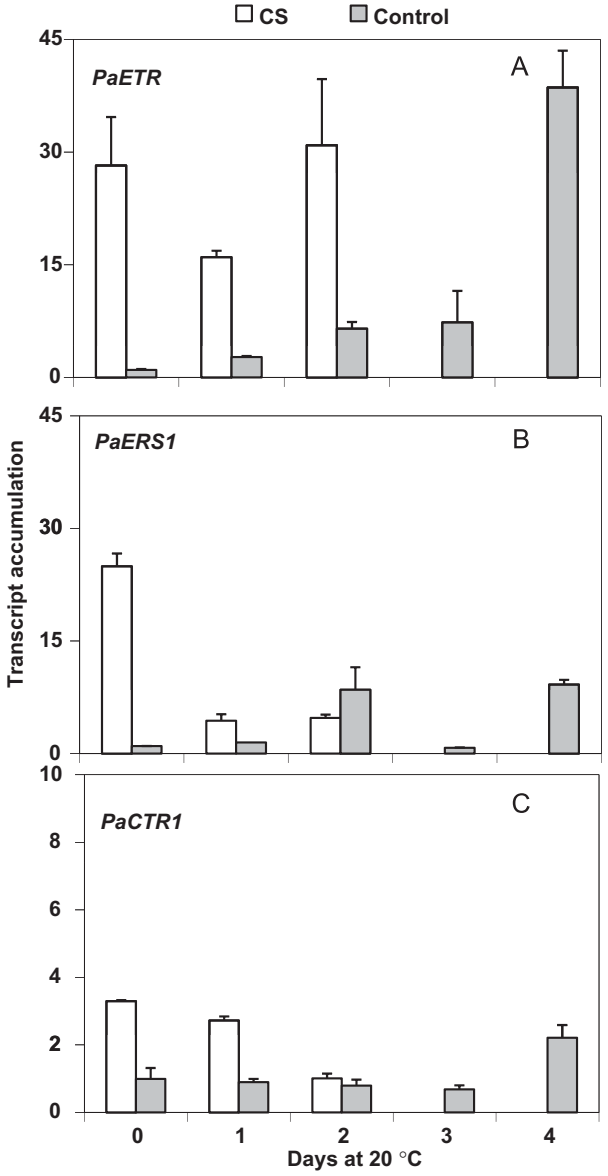

Figure 4. Transcript accumulation of avocado cv. Arad ethylene receptors *PaETR* (A), *PaERS1* (B), and signal transduction element *PaCTR1* (C) in chilling stressed (CS) and control fruit after harvest during 2–4 d of storage at 20 °C. Values have been normalized to control on day 0, arbitrarily set to 1. Vertical bars represent SD.

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with the beginning of the climacteric rise during ripening (Owino et al., 2002). The inability of most avocado varieties to produce ethylene as long as they are attached to the tree results mainly from repression of ACS activity (Sitrit et al., 1986). This repression could be removed by in-orchard chilling stress, resulting in the start of fruit ripening due to an increase in ethylene (Figure 1A). It is possible that on-tree chilling stress caused inhibition of water uptake from the parent plant to the attached fruit (Chinnusamy et al., 2007). Water stress was shown to increase ethylene biosynthesis ACS and ACO genes in the calyx of young persimmon fruit modulated by water loss from the fruit (Nakano et al., 2003). In the orchard plot in which chilled nights occurred, there were fruits on the ground below the tree that was abscised earlier because of chilling stress (data not shown). Abscission of fruits is a well-known phenomena that occurs due to various field stresses, which induce expression of genes modulated by ethylene-signaling (Malladi and Burns, 2008). Also, it was shown that small amounts of ethylene caused a significant drop in abscission of avocado fruitlets (Davenport and Manners, 1982).

Expression of genes involved in ethylene perception and signal transduction

It is widely appreciated that both ethylene biosynthesis and ethylene perception contribute to the regulation of ethylene responses in plant tissues. Our data demonstrate that chilling stress significantly induced levels of transcript accumulation of both *PaETR* and *PaERS1* genes (Figure 4A and B). Similar up-regulation effects of cold treatment were found in pear (El-Sharkawy et al., 2003). The expression levels of both *PaETR* and *PaERS1* in CS fruit on the day of harvest were 25 times higher than the control (Figure 4A and B). In control non-chilled avocado fruit, *PaETR* and *PaERS1* transcripts were low at time 0, up-regulated at the onset of normal ripening, and correlated with the level of climacteric ethylene production (Figure 1A vs. 4A and B). A similar expression pattern of ethylene receptors during ripening was shown in tomato (Klee, 2002), avocado (Owino et al., 2002) pear (El-Sharkawy et al., 2003) and apple (Tatsuki and Endo, 2006). *PaERS1* gene expression was affected by chilling stress more than by ripening (Figure 4B), while *PaETR* gene expression was equally affected by chilling stress and normal ripening (Figure 4A). This indicates that sub-zero temperature activated genes in addition to those activated in normal ripening. The *PaETR* gene from avocado is highly homologous to subfamily-II ethylene receptor genes from other climacteric fruits: the predicted PaETR protein displayed 63% identity to tomato LeETR4, 67% to apple MdETR2, 68% to Chinese pear PpETR2, and 67% to melon CmETR2 protein (Table 2). *LeETR4* transcripts have previously been shown to be up-regulated in ripening fruits (Tieman et al., 2000) and to have a specific role in modulating ethylene responses, including fruit maturation (Kevany et al., 2007). The high *PaETR* and *PaERS1* transcription levels at time 0 in CS fruit may indicate that these genes have already been produced in the avocado fruit on the tree, and the receptor protein degradation manifested in our study by the increase in ethylene receptor transcript levels leads to ethylene production. Ethylene-mediated receptor degradation was demonstrated in tomato changing from the mature

Table 2. Deduced amino acid identity (%) of avocado isolated fragment PaETR (750 aa) with the corresponding regions of ETR sequences of tomato (LeETR4), *Arabidopsis* (AtETR2), apple (MdETR2), Chinese pear (PpETR2), and melon (CmETR2).

Gene	LeETR4, AAD31396	AtETR2, AAC62208	MdETR2, ABI58286	PpETR2, BAD61003	CmETR2, BAF91863
PaETR	63	56	67	68	66

Table 3. Deduced amino acid identity (%) of avocado isolated fragment PaCTR1 (207 aa) with the corresponding regions of CTR1 sequences of tomato (LeCTR1), *Arabidopsis* (AtCTR1), apple (MdCTR1), peach (PdCTR1), and plum (PsCTR1).

Gene	LeCTR1, AAR89823	AtCTR1, BAE99212	MdCTR1, ABI58288	PpCTR1, AAY21209	PsCTR1, ABU68270
PaCTR1	90	89	89	91	90

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green to the breaker stage (Kevany et al., 2007). The sequence of a fragment of *CTR1* encoding a predicted polypeptide of 207 amino acid residues was isolated from avocado. The predicted protein revealed strong homology (89–91% identity) to CTR protein from other climacteric fruits and *Arabidopsis* (Table 3). *PaCTR1* transcript expression levels were less affected by chilling stress and ethylene-dependent ripening compared with *PaETR* and *PaERS1* genes; there was a small increase (less than 3-fold) for *PaCTR1* transcript accumulation in CS fruits at time 0, and in control fruits at day 4 (Figure 4C). Published data on the CTR-like genes show a range of expression changes with ripening. For instance, *PcCTR1* expression increased during pear ripening and in response to ethylene treatment (El-Sharkawy et al., 2003).

In conclusion, our present results show that in-orchard chilling stress leads to an increase in ethylene production via stimulation of ethylene biosynthesis and perception genes, causing accelerated ripening and softening processes in storage. Together, our data suggest that chilling stress in the orchard can eliminate endogenous signal/s and might inhibit the transport of a ''tree factor'' leading to avocado ripening on the tree.

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