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Characterizing ethylene pathway genes during the development, ripening, and postharvest response in *Citrus reticulata* Blanco fruit pulp

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Abstract: Ethylene is involved in the regulation of the natural process of fruit pulp development and postharvest storage. The present study was carried out to determine the expression of eight genes encoding ethylene biosynthesis and signaling during development in Khasi mandarin (*Citrus reticulata* Blanco) fruit pulp as well as during postharvest storage in the fruit pulp (at 4 °C and 20 °C). Gibberellic acid (GA_3) is known to improve internal fruit quality and to mediate various stress responses during postharvest fruit storage in species like citrus. The effect of GA_3 during postharvest storage was also investigated to understand the regulation of the ethylene genes on its own biosynthesis and signaling. The results suggest that 1-*aminocyclopropane-1-carboxylate synthase-2* (*ACS2*), *ethylene response sensor-1* (*ERS1*), *constitutive triple response-1* (*CTR1*), and *ethylene insensitive 3-like-1*(*EIL1*) have a physiological role in the ripening of Khasi mandarin fruit pulp. *1-Aminocyclopropane-1-carboxylate synthase-1* (*ACS1*), *ethylene response-1* (*ETR1*), and *ethylene insensitive-2* (*EIN2*) were found to be developmentally regulated in immature fruit pulp, suggesting their role in rapid growth of the fruit pulp. Postharvest storage at 4 °C and 20 °C influenced the expression of different ethylene-related genes during ripening in Khasi mandarin fruit pulp. Cold storage (4 °C) markedly triggered the transcription of ethylene biosynthetic genes, especially *ACS1* and *ACS2* in the fruit pulp. Exogenous application of GA_3 enhanced cold tolerance especially at the transcript level of *ACS1*, *ACS2*, *ETR1*, and *ERS1* genes in the pulp of Khasi mandarin fruit during cold storage when harvested at the full maturity stage.

Key words: Nonclimacteric, biosynthesis, signaling, maturation, postharvest

1. Introduction

Among fruit tree species, citrus, belonging to the family Rutaceae, is one of the most economically important horticultural fruit crops grown worldwide (Paul et al., 2012). The total annual production of citrus is estimated at over 70 million tons, of which oranges occupy more than half of the total production. It is estimated to reach production of 49.3 million metric tons in 2017/2018 from a previous year (2015/16) production of around 47.06 million metric tons (Statista, 2018). Citrus fruits are well known for their fine flavor and quality as well as their contribution to human health due to their dietary, nutritional, and medicinal properties. They are also good sources of vitamin C, citric acid, and high antioxidant contents like flavonoids, phenolics, pectins, and limonoids (Kumar et al., 2010). Citrus fruits have a very unique physiology and anatomy. They are also known as hesperidium berries, differing from true berries in having a special organization of a very juicy pulp containing vesicles within segments (Cercos et al., 2006). Fruits have been generally classified into two physiological categories as climacteric or nonclimacteric based on the presence or absence of a ripening-related rise in ethylene biosynthesis and respiratory activity (Osorio et al., 2013). Citrus fruits are nonclimacteric in nature, where growth and development follow a 6–12-month period consisting of three stages (Bain, 1958). The earliest stage of fruit growth comprises a cell division stage (stage I) lasting for around 90 days after flowering (DAF). This is followed by a rapid growth period (stage II) starting from 120 DAF (breaker stage in our study) that extends up to 150–180 DAF (Figure 1). During this stage, cell enlargement and water accumulation take place in the fruit pulp. The developing fruit acts as storage sinks during stage II (Cercos et al., 2006). In the final stage (III) starting from 210 DAF until harvest time point, growth is arrested and the fruit undergoes nonclimacteric ripening (Katz et al., 2004). During this stage, external ripening in the fruit takes place by conversion of chloroplast to chromoplast, which is influenced by nutrient availability as well as hormones, especially ethylene (Iglesias et al., 2001). Internal (fruit flesh) ripening in citrus normally takes place at the same time as external ripening (not always) although it does not

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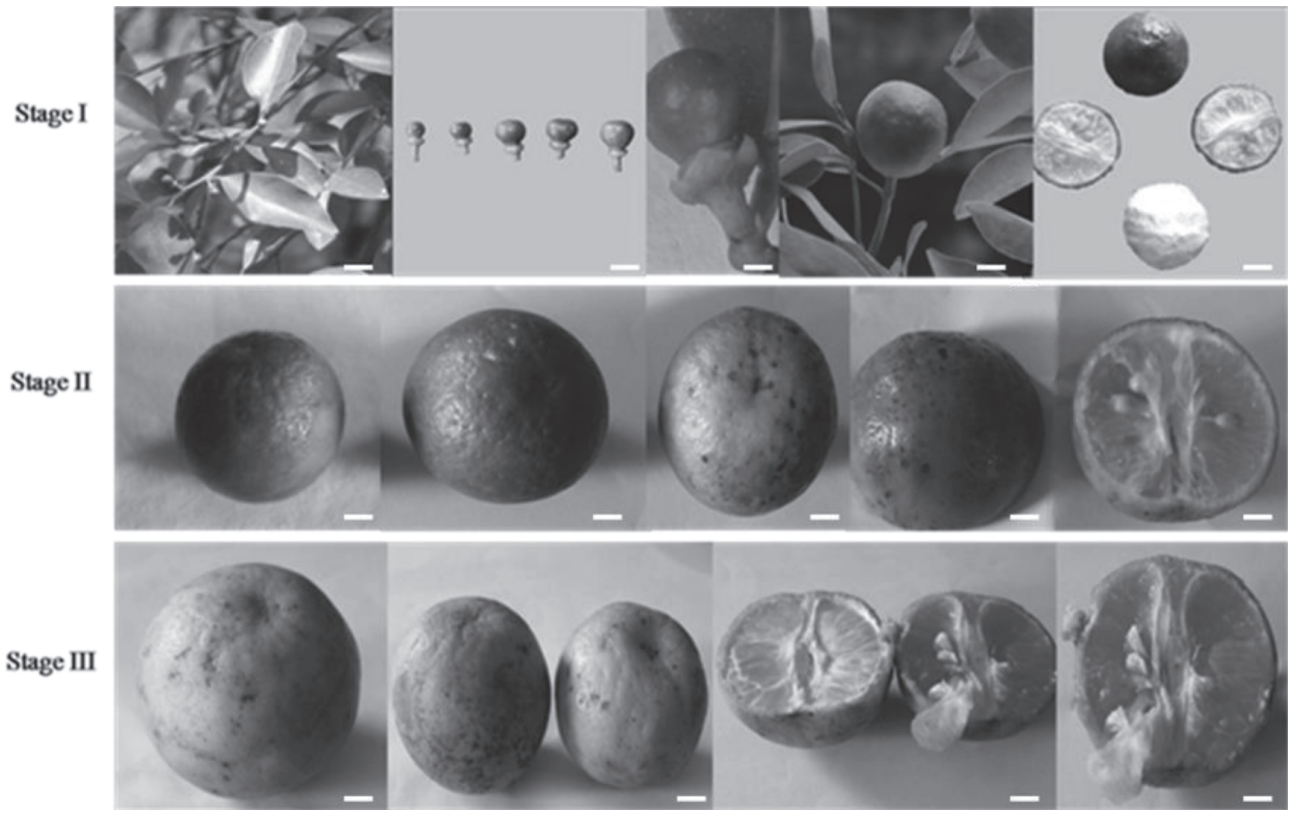

Figure 1. Developmental stages of Khasi mandarin (*Citrus reticulata* Blanco) fruit. Representative images are shown. The scale at the bottom right of the pictures in stage I (first two images from L to R) is 0.25 cm and remaining is 0.5 cm and in stage II and stage III is 1 cm.

seem to be controlled particularly by any hormone. At the end of the maturation process, mature citrus pulp contains a high percentage of water (85%–90%) and other essential components, including carbohydrates, organic acids, vitamin C, and carotenoids (Cercos et al., 2006). Thus, in citrus, there are many aspects of fruit quality that are not acquired only during ripening but also during stage II of fruit pulp development. In contrast to the huge amount of literature available on external ripening, the regulation controlling internal ripening (fruit flesh) is not properly reported in citrus (Cercos et al., 2006).

The different patterns of ethylene biosynthesis observed in nonclimacteric and climacteric fruits are classified into system I and system II (McMurchie et al., 1972). The system I pattern is exhibited by nonclimacteric fruits as well as climacteric fruits in the preclimacteric stage where the rate of ethylene production during ripening is low and they exhibit autoinhibition (Oetiker and Yang, 1995). System II is characterized by autocatalytic rupture of ethylene production associated with the fruit ripening process (McMurchie et al., 1972). At a molecular level, the plant hormone ethylene plays a prominent role in fruit growth and development (Argueso et al., 2007). To understand how ethylene affects fruit development, it is important to consider the pathways related to its biosynthesis and the signaling that initiates the ripening process. The biosynthesis of ethylene occurs through a simple metabolic pathway (Figure 2a) that has been well defined in plants (reviewed previously by Bleecker and Kende (2000)). The initiation of ethylene biosynthesis during fruit growth is marked by conversion of S-adenosyl methionine (SAM) to 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase (ACS). During this conversion, oxidation of ACC to ethylene is carried out by ACC oxidase (ACO) (Alexander and Grierson, 2002). Ethylene is perceived by a family of basic membrane-bound receptors (identified in *Arabidopsis thaliana*) (Figure 2b) that is similar to two-component histidine kinase regulators from bacteria (Schaller and Kieber, 2002). These receptors include ETR1 and ERS1 categorized as subfamily I receptors based on the presence of all the conserved motifs in the histidine kinase domains (John-Karuppiah and Burns, 2010). EIN4, ERS2, and ETR2 are categorized as subfamily II receptors because they lack one or more of the conserved motifs (John-Karuppiah and Burns, 2010). Ethylene signaling begins by binding of ethylene at these receptors. Other

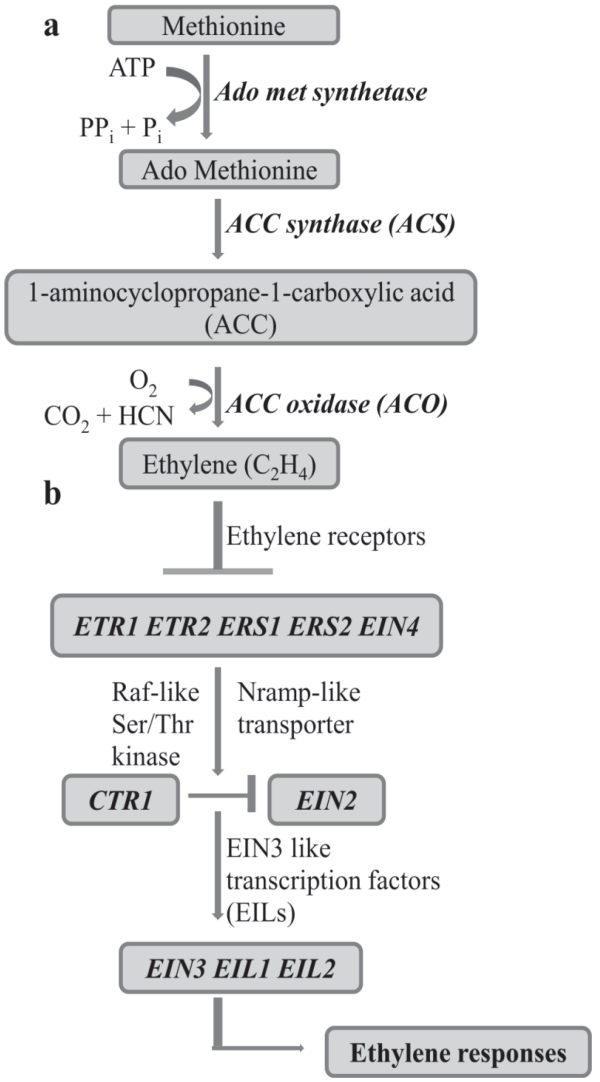

Figure 2. Schematic representation of the ethylene biosynthesis pathway (a) where AdoMet: S-adenosyl-methionine; Met: methionine; and signaling pathway (b) where predicting transmembrane structures are shown as lines in the figure. Adapted from (Chen et al., 2005; Argueso et al., 2007).

receptors such as EIN2 act as positive regulators (Chen et al., 2005) while CTR1 acts as a negative regulator (Chen et al., 2005) in the ethylene signaling pathway. Downstream of EIN2 is a small family of EIN3-like transcription factors (EILs) that act as modulators of ethylene responses and eventually activate ethylene-responsive genes (Chao et al., 1997). The fundamental mechanisms of ethylene-mediated ripening in fruit pulp has been extensively studied in climacteric fruits (as reviewed by Giovannoni (2004)) and limited studies have been reported in a few citrus species (Yu et al., 2012; Wu et al., 2016). Moreover, the characterization of the key family members of ethylene biosynthesis and the signaling pathway that are more important for many biological responses induced during the natural process of development and ripening has not been well reported to date in *Citrus reticulata* fruit pulp.

Postharvest storage in citrus appears to be under the management of an association of regulatory metabolic signals and depends on different factors such as storage temperatures or ripening retardant hormones such as gibberellic acid (GA_3) (Alos et al., 2006). The action of ethylene on fruit postharvest can be inhibited by GA_3 by blocking the receptor sites that continuously form on the plant cell and not allowing ethylene to bind to the receptor sites (Alos et al., 2006). Affinity for the ethylene receptor sites is shown more by GA_3 than by ethylene. GA_3 is known to improve internal fruit quality and shelf-life by maintaining internal hormonal balance during postharvest fruit storage (Bons et al., 2015). GA_3, which controls important physiological processes in plant development, is known to mediate various stress responses in certain plant species such as citrus (Rokaya et al., 2016). GA_3 responses to stress-induced tolerance postharvest have been extensively studied in citrus peels (Porat, 2001; Alos et al., 2006) but far fewer reports are available on the fruit flesh. The evolution of ethylene promotes fruit ripening in the pulp (though minimal) during postharvest storage (Pech et al., 2013). Therefore, treatment with GA_3 could be an interesting means to study the role of the ethylene hormone in regulating its own biosynthesis and signaling network.

The aim of the current study was to report the results from analysis of the expression of genes encoding the ethylene biosynthetic and signaling pathway, such as *1-aminocyclopropane-1-carboxylate synthase-1* (*ACS1*), *2* (*ACS2*), *1-aminocyclopropane-1-carboxylate oxidase* (*ACO*), *ethylene response-1* (*ETR1*), *ethylene response sensor-1* (*ERS1*), *constitutive triple response-1* (*CTR1*), *ethylene insensitive-2* (*EIN2*), and *ethylene insensitive 3-like-1* (*EIL1*) in Khasi mandarin (*Citrus reticulata* Blanco) fruit pulp, a high-quality crop of major commercial importance in the citrus industry of India. The study was carried out throughout the developmental stages until ripening in the fruit pulp and during postharvest storage at 4 °C and 20 °C. The effect of GA_3 treatment during postharvest storage at 4 °C was also studied in the fruit pulp to investigate the involvement of ethylene in the regulation of its own biosynthesis and signaling cascade.

2. Materials and methods

2.1. Plant material and postharvest fruit treatments

Fruit of Khasi mandarin was collected from mature trees growing in a local orchard located in Kamrup (25°97′N and 91°23′E), Assam, under normal culture practices. Fruit at all three stages of development and maturation

was collected at regular intervals from April to November. Sampling stages were 7, 30, 60, 90, 120, 150, 180, and 210 DAF with the last sampling date set at full maturity, that is, after 210 DAF (which is taken as the harvest time point, used as the control). Fruit was collected from three trees (biological replicates), three representative oranges from each tree, for a total of nine representative oranges per sampling stage. After separating the pulp from the peel, the pulp tissue was sliced, frozen in liquid nitrogen immediately after each sampling stage, and ground to fine powder and each of the replicate samples was stored at –80 °C until used. At the harvest time point, biochemical parameters like total soluble solids, titratable acidity, and sugar–acid ratio were measured in the fruit pulp. At this stage, a portion of the sample was used for extracting total RNA; another aliquot was used for the determination of these biochemical parameters. Additionally, these biochemical parameters were measured throughout development until ripening in the Khasi mandarin fruit pulp across all stages (data unpublished). TSS was measured in the fruit pulp at the harvest time point using the anthrone method as described by Hansen and Moller (1975). Sugar–acid ratio and titrable acidity in the fruit pulp were measured by titration (Lacey et al., 2009). Details of these parameters are presented in the Supplementary Table.

In order to study postharvest expression, fruit was harvested at the full maturity stage (after 210 DAF) and segregated into three groups, two with different temperature treatment and the third with hormone and temperature treatment. The first and second groups of fruit were stored for up to 6 weeks at 80%–85% relative humidity at two different temperatures, 4 °C (chilling) and 20 °C (nonchilling), and the gene expression data were analyzed after 1 week (day 7), 3 weeks (day 21), and 6 weeks (day 42). The third group of fruit was treated with GA_3 (Sigma-Aldrich, Mumbai, India) at 60 µM concentration and was stored for up to 6 weeks at 4 °C and the gene expression data were analyzed after 1 week (day 7), 3 weeks (day 21), and 6 weeks (day 42). For the postharvest study, additional fruit was collected from the three trees (biological replicates), 10 representative oranges from each tree, for a total of 30. Ten oranges were used for each treatment during the storage periods. The first, second, and third groups of fruit were divided into three replicates of three oranges each from a total of 10 oranges per treatment and per storage period. Each of the replicate samples was stored individually in plastic containers and was maintained in the same location as the stored space to ensure exposure to identical conditions. Fruit harvested after 210 DAF on day 0 of the treatments was used as the control. The pulp tissue was sliced, frozen in liquid nitrogen immediately after each treatment and storage period, and ground to fine powder, and all of the replicate samples were stored at –80 °C until used.

2.2. Total ribonucleic acid (RNA) isolation and cDNA synthesis

Total RNA was isolated from 5–8 g of citrus fruit pulp by SDS-phenol/chloroform extraction as described by Tao et al. (2004). The yield and purity of RNA were measured using the $A_{260/280}$ and $A_{260/230}$ ratio in a NanoDrop spectrophotometer (Invitrogen, Waltham, MA, USA). First-strand cDNA was synthesized from 1 µg of RNA using Superscript III Reverse Transcriptase (Invitrogen, Life Technology, Mumbai, India) and oligo $(dT)_{12–18}$ primers (Thermo Scientific, Mumbai, India) in a 20-µL reaction volume as per the manufacturer's protocol. A semiquantitative PCR was carried out in a 50-µL reaction each containing 20 ng of cDNA, 1X PCR buffer with 1.5 mM of magnesium chloride, 0.2 mM dNTPs, 0.2 µM each of forward and reverse primers, and 0.5 U Taq DNA polymerase (Bangalore GeNei, India) for optimization of annealing conditions. The reactions were conducted in a

Table. Primer sequences with length of amplicons used for qRT-PCR analysis in Khasi mandarin fruit pulp.

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Amplicon size after sequencing (bp)
ACS 1	TTCGAATCCACTAGGCACAACTT	CAACGCTCGTGAACTTAGGAGA	138
ACS 2	GATGGCGTTATGGCAAGTGA	GAGCAATTTCCATCGTCCGA	134
ACO	AAGATGGCCAGTGGATTGATG	TCACCGAGGTTGACAACAATG	60
ETR 1	TCGTCAGCAGAATCCTGTTGG	GGCCTTAATCTTGCTACTGGACA	120
ERS 1	TTGTGGACTGACTCACTTCATAAGC	ATGACACAAAAGCACAAGCC	102
CTR 1	GTGGATGGCACCGGAAGTT	GAATTTCTCCAAGGTTTTTGCAG	114
EIN 2	GAAAAGGATGATGATGAAGCAGATT	GAAGCCGGACCATCAGACAT	84
EIL 1	ACAGAGCAAGAGTAAGGAATGTGTTG	TCTTGTGCCCGAGACATCTTC	90
GAPDH	GGAAGGTCAAGATCGGAATCAA	CGTCCCTCTGCAAGATGACTCT	75

Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following conditions: initial denaturation at 94 °C for 4 min followed by 35 cycles of final denaturation at 94 °C for 30 s, annealing at 59 °C for 45 s, and initial extension at 72 °C for 2 min with final extension of 72 °C for 7 min. The products were resolved on 2% agarose gel in 1X TAE buffer and the amplicons were detected by staining with ethidium bromide (10 mg/mL, Bio-Rad, India) and photographed using a UVitech gel documentation system (Bangalore GeNei, India). Molecular weights of the PCR products were estimated by comparing them with ØX174/*Hae*III DNA digest and 100 bp DNA ladder.

2.3. Cloning and sequencing of ethylene biosynthetic and signaling genes

The PCR products (amplified cDNAs) were cloned by ligation into a pGEM-T Easy vector (Promega, Madison, WI, USA). The sequences of insert cDNA were confirmed by sequencing the plasmid DNA with T7 forward promoter primer. Sequencing was done to confirm the sequence identity of the amplicons as the primers used for amplification in the study were obtained from *Citrus sinensis*.

2.4. Real-time amplification procedures and quantitative real-time RT-PCR data analysis

Expression profiles for each of the genes were analyzed by quantitative real-time PCR (qRT-PCR) using 2X SYBR Premix Ex Taq (Tli RNaseH Plus; Takara, Clontech Laboratories, Mountain View, CA, USA). For qRT-PCR, 1 µL of cDNA was used in 20-µL reaction volumes. The reactions were conducted in a real-time PCR cycler (Rotor-Gene Q System, QIAGEN, Hilden, Germany) with the following conditions: preincubation at 95 °C for 5 min followed by 40 cycles of 30 s at 95 °C for denaturation, 30 s at 59 °C for annealing, and 45 s at 72 °C for elongation (signal acquiring stage). The primers used for qRT-PCR were those reported by John-Karuppiah and Burns (2010). The sequences of the primer pairs used in the study and the length of amplicons obtained are listed in the Table. In the present study, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a reference gene (Wu et al., 2014a) to normalize the variation in the concentration of template that may have occurred in each replicate. The forward and reverse sequences for the primer used for the *GAPDH* gene are also listed in the Table. For the selection of a reference gene, two potential housekeeping genes, *GAPDH* and *ACTIN* (*ACT*), were used based on previous reports (John-Karuppiah and Burns, 2010; Rodrigo et al., 2013). The software BestKeeper was used to test the stability of these genes (Pfaffl et al., 2004). It was observed that *ACT* showed high variability to the measurements and generated irreproducible results [SD ± crossing point (CP) = 0.58]. On the other hand, acceptable reproducibility of the data was obtained with *GAPDH* (SD ± CP = 0.39) (Alos et al., 2014; Wu et al., 2014a). Hence, for the present study, *GAPDH* was selected and used. Relative gene expression was calculated by the comparative ΔCt method (Livak and Schmittgen, 2001). For every sample, the Ct (threshold cycle for target amplification) value for the gene of interest was subtracted from the Ct value of *GAPDH* (ΔCT_1). In control replicates, the Ct value for the gene of interest was subtracted from the Ct value of *GAPDH* (ΔCT_2). ΔΔCT was calculated by subtracting the ΔCT of control replicates from the ΔCT of all individual replications for all stages at each sampling point (ΔCT_1-ΔCT_2). Relative gene expression was calculated using the equation $2^{-(\Delta\Delta CT)}$. Relative quantification was carried out by constructing standard curves for each target gene and for the endogenous control by making a 10-fold dilution series of an RNA sample to check whether the target genes and *GAPDH* had similar efficiency of amplification. The dissociation curve generated after the amplification showed that a single amplicon was produced in each reaction. For each gene, the expression level in the fruit pulp at the harvest time point (control) was arbitrarily assigned an induction value of 1-fold. Final expression values refer to the fold increase in relation to the control.

2.5. Statistical analysis

Experimental data are the means ± SD of three replicates of the determinations for each sample. Standard deviations of the mean were calculated using Microsoft Excel and are represented as vertical bars in the figures. Analysis of variance tests were performed and the Tukey test was carried out using the software GraphPad Prism (GraphPad Software, San Diego, CA, USA) to compare the statistical significance of differences in gene expression between the samples and control fruit pulp. Here, $P < 0.05$ was considered to be statistically significant, $P < 0.01$ was considered to be more statistically significant, and $P < 0.001$ was considered to be extremely statistically significant.

3. Results

3.1. Sequence analysis of cloned inserts

The sequence analysis showed that the *ACS1* sequence obtained had homology with the partial length sequence of *Citrus sinensis* 1-aminocyclopropane-1-carboxylate synthase 1-like mRNA (LOC102621630, 1834 bp) in the NCBI database. Similarly, the nucleotide sequence of *ACS2* showed similarity to the partial length sequence of *Citrus sinensis* mRNA for ACC synthase (AJ012696, 1186 bp). The sequence of *ACO* showed similarity to a partial length sequence of *Citrus sinensis* ACC oxidase (LOC102577997, 1217 bp) mRNA. The *ETR1* sequence showed similarity to a partial length sequence of *Citrus sinensis* ethylene response 1 (*ETR1*), transcript variant X1, mRNA (3052 bp). The *ERS1* sequence showed similarity to a partial

length sequence of *Citrus sinensis* ethylene response sensor 1, transcript variant X3, mRNA (LOC102577971, 2357 bp). The sequence of *CTR1* showed similarity to a partial length sequence of *Citrus clementina* hypothetical protein (CICLE_v10015962mg, 1308 bp) mRNA complete cds and the *EIL1* sequence showed similarity to a partial length sequence of *Citrus sinensis* ethylene-insensitive 3-like 1 protein (*EIL1*), mRNA (2296 bp). *EIN2* was obtained from the NCBI database and showed similarity to a partial length sequence of *Triticum turgidum* subsp. durum *CMO* gene for choline monooxygenase, cultivar Ofanto, clone (TdCMO3b2, 1156 bp).

3.2. Expression of ethylene biosynthetic genes during fruit pulp development and ripening

In the fruit pulp, the transcript level of two ethylene biosynthetic genes (*ACS2* and *ACO*) at 7 DAF was comparable to that found in the fruit pulp at the harvest time point (control), while it had higher expression in the case of the *ACS1* gene. Similarly, the transcript level of the *ACO* gene in the fruit pulp at 30 DAF and 60 DAF was comparable to that found in the fruit pulp at the harvest time point, while it had higher gene expression in the case of the *ACS1* gene. Additionally, the transcript level of the *ACS2* gene in the fruit pulp at 210 DAF was comparable to that found in the fruit pulp at the harvest time point, while it had higher gene expression in the case of the *ACO* gene. The pattern of expression of the three ethylene biosynthetic genes (*ACS1*, *ACS2*, and *ACO*) appeared quite intriguing during Khasi mandarin fruit pulp development. From 7 DAF to 30 DAF, transcript levels of *ACS1* in the fruit pulp were upregulated (about 6-fold and 10-fold that of the harvest time point), being statistically significant ($P < 0.001$). The transcript levels had minimum expression from the breaker stage (120 DAF of stage II) towards maturation stage III until the harvest time point (Figure 3a). However, a different pattern of expression was observed with the *ACS2* gene during the same development period, where the transcript level of *ACS2* was lower until the middle of stage II (120–150 DAF) of fruit pulp development. At 180 DAF, *ACS2* transcript level was upregulated (about 1.8-fold that of the harvest time point), after which the level of upregulation slightly decreased until the harvest time point (Figure 3b). These changes were statistically significant ($P < 0.001$ and $P < 0.05$). The transcript level of *ACO* in the fruit pulp was found to be expressed throughout all the stages (I, II, and III) of development until the harvest time point, with a transient rise at 210 DAF (upregulated by about 7.0-fold that of the harvest time point), after which the expression level dropped at the harvest time point (Figure 3c).

3.3. Expression of ethylene signaling genes during fruit pulp development and ripening

The transcript level of the *ETR1* receptor gene in the fruit pulp at 30 DAF was comparable to that found in the fruit pulp at the harvest time point, while it had higher gene expression in the case of the *CTR1* and *EIN2* genes. Similarly, the transcript level of the *ERS1* gene in the fruit pulp at 180 DAF was comparable to that found in the fruit pulp at the harvest time point, while it had higher gene expression in the case of the *CTR1* and *EIN2* genes and much higher expression in the case of the *EIL1* gene. The transcript levels of the *ETR1* gene in the fruit pulp were highest at the onset of development in stage I at 7 DAF (upregulated by about 1.9-fold that of the harvest time point), statistically significant at $P < 0.001$ (Figure 3d). After this, the transcript levels were downregulated, showing constant expression until maturation with a transient increase at the harvest time point. Transcript levels of the *ERS1* gene were elevated in the fruit pulp at the breaker stage (120 DAF, to more than 3.0-fold increase that of the harvest time), statistically significant at $P < 0.01$, after which it was expressed until the harvest time point (Figure 3e). On the other hand, the *CTR1* gene in the fruit pulp was upregulated throughout development until the harvest time point but there were many variations in the level of upregulation. The transcript level was observed to be significantly highest at 7 DAF (upregulated by about 15-fold that of the harvest time point), after which there was a sharp drop in the level of upregulation at 30 DAF (upregulated by about 1.9-fold), followed by many fluctuations until the harvest time point (Figure 3f). The transcript level of the *EIN2* gene in the fruit pulp was noted to be significantly high at 7 DAF (upregulated by about 8.0-fold that of the harvest time point) and maintained more or less similar levels until harvest time (Figure 3g). Transcript levels of *EIL1* were very low during stage I of fruit pulp development (7–90 DAF), followed by a sharp increment during stage II and stage III (between 120 and 210 DAF, statistically significant at $P < 0.001$), which dropped towards harvest time (Figure 3h). A higher expression level was shown by the *EIL1* gene in the fruit pulp at the breaker stage (120 DAF, increased by about 6.0-fold that of the harvest time point).

3.4. Effect of postharvest storage temperature and GA_3 hormone treatment on mRNA expression of ethylene biosynthetic genes

The expression of the ethylene biosynthetic genes was further studied in the fruit pulp harvested at the full maturity stage (after 210 DAF) and subjected to temperature treatments. Cold storage at 4 °C induced expression of the *ACS1* gene in the fruit pulp significantly from week 1 until week 6 during postharvest storage. The level of induction in *ACS1* was lowest in week 1 with a sharp rise in week 3 and then gradually increased until week 6 of storage (upregulated by about 1.7-, 9.0-, and 11.5-fold). On the other hand, GA_3 treatment also induced expression of the *ACS1* gene in the fruit pulp at 4 °C from week 1 until

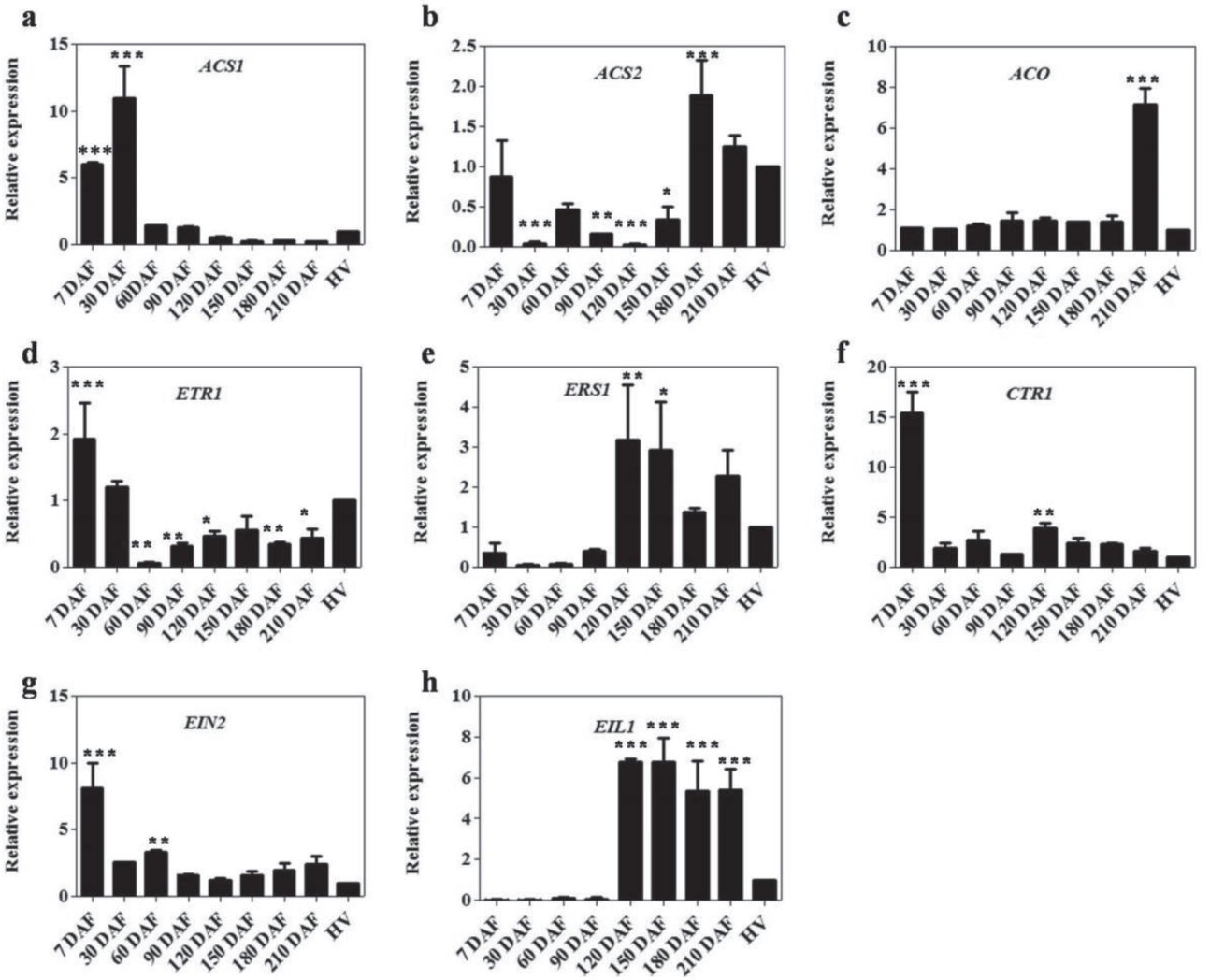

Figure 3. Expression of different ethylene biosynthesis (a, b, and c) and signaling genes (d, e, f, g, and h) in fruit pulp of Khasi mandarin during development and maturation. HV: Harvest; DAF: Days after flowering. The *y*-axis shows the relative gene expression levels (fold change) analyzed by qRT-PCR, which were plotted against the developmental stages (*x*-axis). For each gene, an arbitrary value of 1-fold was assigned to the expression determined in the fruit pulp at harvest time point (control). Bars represent the mean ± SD of n = 3 biological replicates. Statistical significance analyzed using GraphPad t-test online software was represented by a single asterisk (significant at $P < 0.05$), double asterisk (significant at $P < 0.01$), and triple asterisk (significant at $P < 0.001$).

week 6 of postharvest storage but the level of expression at weeks 3 and 6 was lower in GA_3-treated fruit pulp at 4 °C compared to fruit pulp only at 4 °C (upregulated by about 1.3- and 9.5-fold) (Figure 4a). Similarly, cold storage at 4 °C induced expression of the *ACS2* gene in the fruit pulp gradually from week 1 until week 6 of storage at 4 °C (upregulated by about 1.4-, 1.5-, and 1.7-fold). GA_3 treatment also induced expression of the *ACS2* gene in the fruit pulp at 4 °C gradually from week 1 until week 6 of storage but again there was a repressive effect of GA_3 in *ACS2* since expression was lower in GA_3-treated fruit pulp at 4 °C compared to fruit pulp only at 4 °C (upregulated by about 1.0-, 1.1-, and 1.2-fold). These changes were statistically significant ($P < 0.05$, Figure 4b). Cold storage at 4 °C induced expression of the *ACO* gene in the fruit pulp significantly only by week 6 (upregulation of more than 1.5-fold). GA_3 treatment also resulted in a downward trend of the *ACO* gene in the fruit pulp at 4 °C during weeks 1 and 3 but expression was induced significantly by week 6 of postharvest storage (upregulated by about more than 5-fold) (Figure 4c).

During storage at a nonchilling temperature (20 °C), it was observed that the *ACS1* gene expression in the fruit pulp accumulated until week 1 (upregulated by about 1.3-fold) but declined over the period of storage (weeks 3 and 6; downregulated by about 0.1-fold). Accumulation

of transcripts of the *ACS2* gene declined during storage at 20 °C, where the transcripts were found to be significantly low, maintaining similar levels until week 6 of storage (downregulated by about 0.04-fold). The accumulation of *ACO* transcripts declined initially at 20 °C but had accumulated significantly by week 6 of postharvest storage ($P < 0.01$) (Figure 4c).

3.5. Effect of postharvest storage temperature and GA_3 hormone treatment on mRNA expression of ethylene signaling genes

During cold storage at 4 °C, expression of *ETR1* and *ERS1* genes in the fruit pulp was very low until week 3 but was markedly stimulated by week 6 of postharvest storage (upregulated by about 8.0- and 9.5-fold). GA_3 treatment also resulted in decreased expression of *ETR1* and *ERS1* genes in the fruit pulp at 4 °C until week 3 of storage but was stimulated by week 6 of storage (upregulated by about 1.5- and 9.0-fold) (Figures 4d and 4e). However, the level of expression was reduced as compared to storage at 4 °C. During storage at 20 °C, expression of *ETR1* and *ERS1* genes was significantly increased from week 3 until week 6 of storage (upregulated by about 2.5- and 6.3-fold and 7.0- and 9.0-fold, $P < 0.05$). During cold storage and GA_3 treatment followed by storage at 4 °C, transcript levels of the *CTR1* gene were maintained at low levels until week 6 of storage (downregulated by about 0.9- and 0.95-fold). A similar pattern of expression was also observed at 20 °C except that it showed a sharp rise at week 6 of storage (upregulated by about 9.0-fold) (Figure 4f).

In the case of the *EIN2* gene, expression levels were continuously downregulated in the fruit pulp at 4 °C and GA_3 treatment followed by storage at 4 °C during the three storage periods (Figure 4g). However, storage at 20 °C induced expression significantly at week 6 of storage (upregulated by about more than 7.5-fold) ($P < 0.05$). Similarly, cold storage and GA_3 treatment followed by storage at 4 °C did not stimulate the expression levels of the *EIL1* gene in the fruit pulp during the three storage periods. However, storage at 20 °C induced its expression only during weeks 3 and 6 of storage (upregulated by about 1.4- and 2.5-fold) (Figure 4h).

4. Discussion

Ethylene has long been known to play a vital role in the maturation process of climacteric fruits (Alexander and Grierson, 2002). However, in nonclimacteric fruits, even the lowest levels of ethylene available during maturation have possible implication in the development and ripening process (Trainotti et al., 2005). In the present study, we observed that *ACS1* transcripts were expressed in immature young Khasi mandarin fruit pulps (stage I) but were not expressed in mature fruit pulps (stages II and III). This followed an autocatalytic transition from system II to system I (Katz et al., 2004). Similarly, the *ACS2* gene was also developmentally regulated, however, showing a climacteric-like transition from system I to system II (Katz et al., 2004). A similar study was reported by Katz et al. (2004), in the Valencia orange (*Citrus sinensis* L. Osbeck), where *CsACS1* was observed to be involved in citrus system II-like behavior of ethylene biosynthesis, whereas *CsACS2* was apparently involved in system I. A transitory increase in ethylene evolution has also been established to occur in nonclimacteric grapes just at the onset of ripening (Chervin et al., 2004). On the other hand, the *ACO* gene was found to be constantly expressed in both systems I and II but is highly regulated in system II like ethylene biosynthesis in Khasi mandarin fruit pulps. This result is consistent with the observations made by Katz et al. (2004) and Wu et al. (2014b), where expression of the *CsACO* gene was constantly upregulated in sweet orange (*Citrus sinensis* L. Osbeck) cultivars throughout the developmental stages. Similarly to our study, Trainotti et al. (2005) reported high expression of the *FaACO1* gene in nonclimacteric strawberry in ripe red fruit as compared to unripe fruit.

In the present study, the *ETR1* receptor gene showed a significant increase in transcript levels during early development in the fruit pulps that was reduced during maturation with a transient rise at the harvest time point, suggesting that this gene might be associated with rapid growth of the fruit pulp in nonclimacteric citrus fruits. However, in a previous study, the *CsETR1* gene was observed to be constantly expressed in Valencia oranges (*Citrus sinensis* L. Osbeck) throughout the fruit developmental stage (Katz et al., 2004). In the present study, the expression of the *ERS1* receptor gene was observed to be highest in the mature fruit pulps, showing a continuous increase during the ripening phase until the harvest time point. This finding might suggest the importance of *ERS1* in the ripening of citrus fruit pulp. The present result also substantiates the difference in *ERS1* gene expression between immature and mature citrus fruit pulp, which suggests the role of the *ERS1* gene in the modulation of differential sensitivity to ethylene in citrus fruit (Katz et al., 2004). The increase in transcript levels of ethylene receptors in fruit ripening has been stated to be a natural response in opposition to increased ethylene biosynthesis for protecting the homeostatic equilibrium and as a means of regulating ethylene responses (Klee et al., 2003). Furthermore, *CTR1* and *EIN2* genes were observed to be continuously expressed throughout development until harvest. However, the *EIN2* gene was found to be more responsive in the early stages of development in the fruit pulp, while variation in transcript levels was observed with the *CTR1* gene throughout fruit pulp development. This suggests that *CTR1* and *EIN2* might be triggered

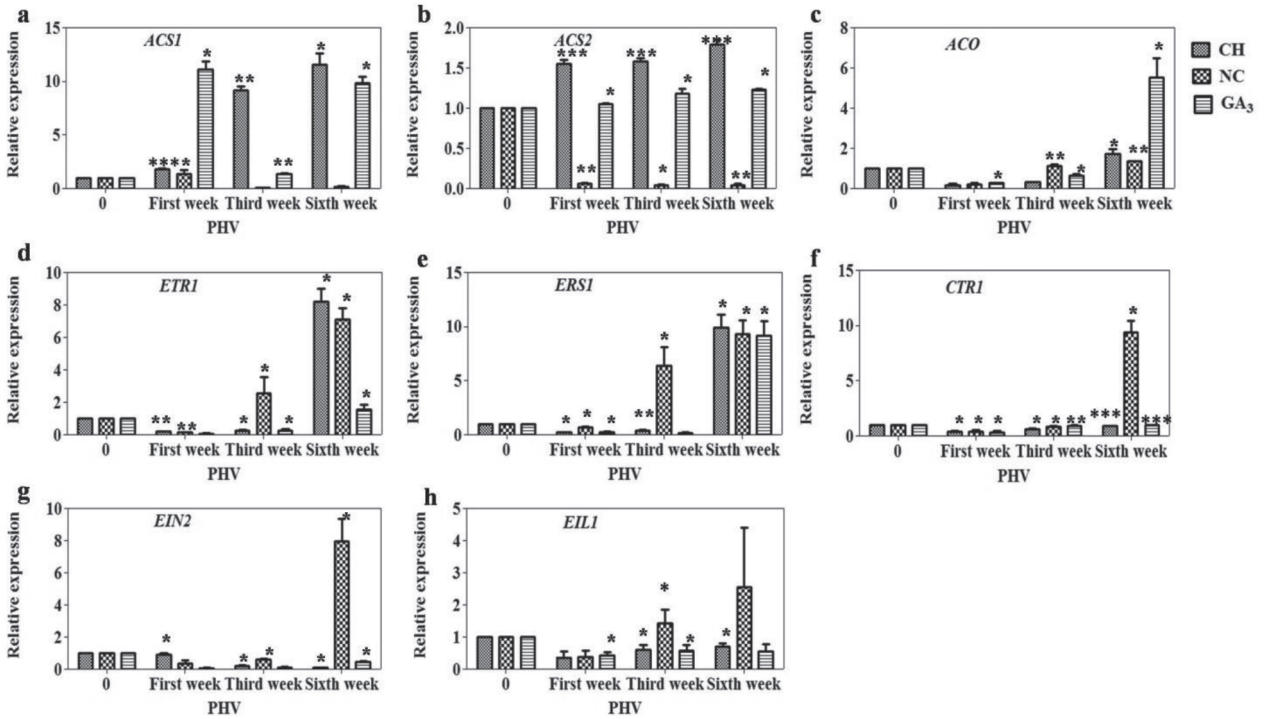

Figure 4. Expression of different ethylene biosynthesis (a, b, and c) and signaling genes (d, e, f, g, and h) in fruit pulp of Khasi mandarin exposed to chilling (4 °C), nonchilling (20 °C), and GA_3 treatment at 4 °C during the first week, third week, and sixth week of postharvest storage. CH: Chilling; NC: Nonchilling; GA_3: Gibberellic acid treated; PHV: Postharvest. The *y*-axis shows the relative gene expression levels (fold change) analyzed by qRT-PCR, which were plotted against the storage periods (*x*-axis). For each gene, an arbitrary value of 1-fold was assigned to the expression determined in the fruit pulp on day 0 of the treatments (control). Bars represent the mean ± SD of n = 3 biological replicates. Statistical significance analyzed using GraphPad Prism software was represented by a single asterisk (significant at $P < 0.05$), double asterisk (significant at $P < 0.01$), and triple asterisk (significant at $P < 0.001$).

even by the slightest amounts of ethylene available during the maturation of citrus fruit pulp. Similar results were reported in sweet orange (*Citrus sinensis* L. Osbeck) by Wu et al. (2014b), where transcript accumulation of *CsCTR1* and *CsEIN2* genes was constant over the ripening stages despite a slight peak at 170 DAF in the midripening phase. In the present study, transcript abundance of the *EIL1* gene increased during fruit pulp enlargement and maturation, suggesting their physiological role in the ripening of citrus fruit pulp. Additionally, the fact that the *EIL1* gene is reabundant during maturation stages might suggest some interesting cues for further exploration of relations between ethylene and the ripening of citrus fruit pulp. Likewise, transcripts levels of *CsEIL* were reported to accumulate more during the late-ripening stages in sweet orange (*Citrus sinensis* L. Osbeck) fruit pulp, suggesting that *CsEIL* plays a role in determining ethylene sensitivity during citrus fruit pulp ripening (Wu et al., 2014b). Our results also highlight that the basal level of ethylene available during ripening in Khasi mandarin fruit pulp may be sufficient to induce considerable changes in the downstream regulation of ethylene receptors. This indicates that ethylene signals are potentially still active during the maturation stage in the fruit pulp and that the inhibition of ethylene signaling may also severely impact ripening in citrus fruit pulp.

The present study was also carried out to further understand the regulation of ethylene biosynthesis and signaling during postharvest storage in Khasi mandarin fruit pulp. It was observed that during cold storage (4 °C) *ACS1* and *ACS2* gene expression increased, while *ACO* gene expression initially declined and increased only at the late storage period (week 6), showing that cold directly triggers transcription of *ACS1* and *ACS2* genes, and to a lesser extent in the *ACO* gene. Contrary to this, storage at nonchilling temperature (20 °C) effectively reduced the expression of *ACS1* and *ACS2* genes, but not in the *ACO* gene since there was an increase in the expression of this gene at 20 °C. Thus, the *ACO* gene was partially expressed at low temperature but this constraint was removed during storage at 20 °C as also observed in cold-stored grapefruits (Lado et al., 2015). Results from a previous study on grapefruit (*Citrus paradisi*) cultivar Marsh have shown a similar induction in expression of *ACS* and *ACO* genes

after cold storage (Lado et al., 2014). In the fruit of other citrus varieties such as the Valencia orange (*Citrus sinensis* L. Osbeck) exposed to different temperature conditions, a differential increase in expression of both *ACS* genes was observed, highlighting a response triggered by a specific tissue or induced by specific stress (Katz et al., 2004). Previous studies have also indicated a positive association between cold stress and ethylene biosynthesis as observed in cold-stored fortune mandarin (Zacarias et al., 2003). The results generated in the present study suggest the involvement of chilling temperature as a stimulus inducing activation of the ethylene biosynthesis genes at the transcript level. It further indicates that transfer of the fruit to nonchilling temperature might encourage the ethylene biosynthetic *ACO* gene to reach a more favorable condition and trigger ethylene production. It is interesting that while both *ACS1* and *ACS2* transcripts were cold induced an increase was observed in these transcripts in the pulp of GA_3-treated fruit at 4 °C, but it was not overstimulated. Compared with the fruit stored at 4 °C, fruit treated with GA_3 and storage at 4 °C exhibited lower expression of *ACS1* and *ACS2* genes in the fruit pulp. This might be because of the elevated GA_3 levels in the treated fruit pulp during periods of cold storage following exogenous GA_3 application, which resulted in decreased expression of *ACS1* and *ACS2* genes. This suggested that the elevated GA_3 levels might contribute to enhanced chilling tolerance (Trainotti et al., 2005). This suggests that the turnover rate of ethylene biosynthesis is suppressed at low temperature thereby increasing the efficiency of GA_3 (Asif et al., 2009). Notably, exogenous GA_3 application relatively upregulated the *ACO* gene expression at week 6 of storage as compared to the fruit stored at 4 °C during the same storage period. This might be associated with cold-induced GA_3 deficiency (Figure 4c). This result is consistent with a previous study, which indicated a connection between low temperature and interruption of GA_3 homeostasis in cherry tomato (*Solanum lycopersicum* L. var. *cerasiforme*) during cold when harvested at the mature stage (Ding et al., 2015). Repression of stress-induced ethylene biosynthesis correlates that ethylene suppresses the molecular and biochemical processes controlling its own biosynthesis as was also observed in cold-stored grapefruits (Lado et al., 2014).

ETR1 and *ERS1* gene expression was cold stimulated, especially *ERS1* since its relative expression level was higher in cold-stored fruit pulp compared to that in the *ETR1* gene. They were stimulated only during week 6 (day 42) of postharvest storage, suggesting that temperature does not trigger the response of these genes during postharvest storage of citrus pulp. However, both genes were induced at the nonchilling temperature around week 3 (day 21) and expression was maintained until week 6. This perhaps might result from an increase in ethylene production during storage. A similar relationship has been reported between the increase in ethylene production and the increase in transcript levels of the receptor gene during cold storage of grapefruit (*Citrus paradisi*) cultivar Marsh (Lado et al., 2014). The results indicate that the *ERS1* gene is most receptive to variations in postharvest treatment conditions and these changes in the expression of *ETR1* and *ERS1* genes may be used for determining tissue sensitivity to ethylene. Moreover, in the case of *ERS1* and *ETR1* gene expression, the results indicated that GA_3 treatment showed reduced chilling stress in the fruit pulp at 4 °C during long-term cold storage. Although the transcription of *ETR1* and *ERS1* genes was also induced in response to GA_3 treatment at 4 °C, the increase in the expression of the fruit pulp at 4 °C was higher as compared to the GA_3-treated fruit at 4 °C (Figures 4a and 4b). This suggests that exogenous GA_3 may not control the expression of these genes (Trainotti et al., 2005) during the storage periods as these genes were highly expressed during cold storage. On the other hand, the expression level of the *CTR1* gene was neither cold-induced nor stimulated by GA_3 treatment. However, expression of *CTR1* was strongly induced by nonchilling temperature during the late storage period (week 6), although not during the early storage periods, suggesting its role in the nonchilling response during the postharvest storage of citrus pulp. *EIN2* and *EIL1* genes showed increased expression in response to nonchilling temperature and this was particularly notable for the *EIL1* gene, probably suggesting that these genes might be involved in the cascade of events induced during nonchilling stress in citrus fruit pulp. However, these two genes were not stimulated by chilling temperature or GA_3 treatment, suggesting that they might be positively regulated by ethylene. According to Tian et al. (2000), nonclimacteric fruits can have different ethylene receptors that may have different regulatory functions.

In summary, the findings of the study confirm the role of *ACS2*, *ERS1*, *CTR1*, and *EIL1* genes in the ripening of Khasi mandarin *Citrus reticulata* fruit pulp. We noted that postharvest storage at 4 °C and 20 °C influenced the expression of different ethylene-related genes during ripening in Khasi mandarin fruit pulp. *ACS1*, *ACS2*, and *ERS1* were the most receptive to the postharvest stress response. The results of the study confirm that cold storage induces ethylene biosynthesis in the pulp of Khasi mandarin fruit. Our study suggests that the feedback regulation in chilled Khasi mandarin fruit pulp responded well to the flow in GA_3 content following exogenous application of GA_3 under specific stress conditions. Our results demonstrated that exogenous application of GA_3

enhanced cold tolerance especially at the transcript level of *ACS1*, *ACS2*, *ETR1*, and *ERS1* genes in the pulp of Khasi mandarin fruit during cold storage when harvested at the full maturity stage. The findings of the study provide an insight into the mechanisms by which GA_3 mediates fruit tolerance to chilling stress during long-term cold storage, and it might be possible to develop effective approaches for postharvest chilling stress management of nonclimacteric fruit. This work might act as a baseline to establish a possible role for ethylene in the natural process of ripening in nonclimacteric citrus fruit pulp and help us to understand the effect of temperature on postharvest storage of Khasi mandarin fruit pulp.

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Supplementary Table. Biochemical parameters measured in Khasi mandarin fruit pulp at harvest time point.

Sampling stage	Total soluble solids (%) ± SD	Sugar–acid ratio ± SD	Titratable acidity (g/mL) ± SD
Harvest time point	11.06 ± 0.03 a	10.73 ± 0.78 a	0.31 ± 0.06 b

Means followed by the same letter in each column are not significantly different based on the Tukey test at 5% probability; SD: standard deviation.