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Assessing Shelf-life Ripening of Newly Imported Greenhouse Tomato Cultivars in Saudi Arabia: A qPCR Approach

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WHETHER tomatoes are harvested fresh or stored, their shelf life determines their economic lifetime. At room temperature, fruit firmness is highly associated with shelf life. In tomato fruits (*Solanum lycopersicum* L. cv. 'Myrock'), ethylene is responsible for the loss of firmness associated with climacteric fruit. The ethylene biosynthetic pathway is governed by two families of genes coding as 1-aminocyclopropane-1-carboxylate oxidase (*ACO*) and 1-aminocyclopropane-1-carboxylate synthase (*ACS*). In the current study, the gene expression of the *ACO* gene (*LeACO1*) and the *ACS* gene (*LeACSA*) with a housekeeping gene (*LeGAPDH*) were investigated for their influence on shelf life and ethylene production in three different tomato varieties (Roma, Sakura F1, and GS12), and quantified using the quantitative polymerase chain reaction method (qPCR). The genetic diversity in the molecular regulation for the perception of ethylene biosynthesis during and after fruit ripening was investigated on the three tomato cultivars in three different maturity stages: mature green (MG), breaker (BR), and full red (FR). The results suggested that the *LeACSA* gene ethylene production is responsible for revealing the stage of FR tomatoes in all tested tomato species by the significant accumulation of *ACS* mRNA in the fruit. The expression of the *LeACO1* gene in the tomato variety Roma was decreased more in the full red-ripe (FR) tomato fruits than in the BR and MG stages. However, in Sakura F1, the expression of *LeACSA* and *LeACO1* were decreased in the full red stage, which showed the lowest ethylene production and the most extended shelf life, which could be used in tomato breeding programs with local cultivars to provide a core cultivar with the most extended shelf-life duration.

Keywords: Ethylene Biosynthesis, 1-Aminocyclopropane- 1-carboxylic acid oxidase (ACO), 1 - Aminocyclopropane- 1-carboxylic acid synthase (ACS), Tomato, Shelf-Life, qPCR.

Introduction

In the genus Solanum section Lycopersicon, which includes domesticated tomato varieties, there are also 12 wild relatives (Bai et al., 2007). These wild tomato phenotypes have adapted to local environments and evolved to thrive under varying conditions (Nakazato et al., 2010). The ripening process of tomatoes involves the release of ethylene, climacteric respiration, and various biochemical changes that lead to solubilization of cell wall degradation of synthesis of lycopene and chlorophylls (Ealing, 1994; Sozzi et al., 2001; Atta et al., 2009; Lin et al. 2014). Ethylene, a plant hormone, regulates numerous physiological processes associated with fruit ripening in higher plants, including alterations in flesh texture, skin color, flavor, and aroma variation (Picton et al., 1995; Abd El-Moneim et al 2020).

Fruits can be divided into non-climacteric and climacteric according to the biochemical alterations throughout the ripening. Climacteric fruits undergo an ethylene burst and increased respiration (Giovannoni, 2001, 2004; Chen, 2022). Climacteric fruits, such as red-ripe tomatoes, exhibit increased respiratory rates and ethylene production during ripening, even before the appearance of color (Lü et al., 2018; Klee et al., 2011; Lin et al. 2014; Chen, 2022).

The climacteric nature is observed in several fruits, including apples, bananas, and melons, where the ethylene burst is a key factor in fruit ripening (Ayub et al., 1996; Chen, 2022). While ethylene is the primary inducer of fruit ripening in climacteric fruits, both ethylene-independent and ethylene-dependent pathways coordinate ripening in non-climacteric fruits (Giovannoni, 2001, 2004). The biosynthetic pathway of ethylene involves two enzymes coded by important gene families. The first enzyme is 1-aminocyclopropane-1-carboxylate synthase (*ACS*), which catalyzes the conversion of S-adenosyl-L-methionine (SAM) to the ethylene intermediate 1-aminocyclopropane-1-carboxylic acid (ACC), which is usually the rate-limiting step (Rottmann et al. 1991). The second enzyme is 1-aminocyclopropane-1-carboxylate oxidase (*ACO*), which is correspondingly identified as an ethylene-forming enzyme (EFE) that exchanges *ACC* for ethylene (Bleecker & Kende, 2000). Ethylene stimulates the translation and transcription of ripening-associated enzymes, including pectin

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methylesterase, polygalacturonase, and β-galactosidase (Blumer et al., 2000). Recent research by Wang et al. (2022) has shed light on the atypical climacteric behavior observed during fruit ripening in blueberries. The study demonstrated that both Southern highbush and rabbiteye blueberry varieties exhibit a climacteric ripening pattern characterized by increased respiration rates and ethylene production, peaking around the Pink and Ripe stages of fruit development (Yokotani et al., 2009; Sharma et al. 2021). This finding challenges the traditional classification of blueberries as non-climacteric fruits and highlights the complexity of ethylene-mediated ripening processes in different fruit types. Moreover, the study by Zhou et al. (2021) delves into the regulation of strawberry fruit ripening through N6-methyladenosine (m6A) RNA modification in an abscisic acid (ABA)-dependent manner. While focused on strawberries, the research raises the question of whether mRNA m6A methylation, known to regulate ripening in climacteric fruits like tomatoes, plays a similar role in controlling ripening in other types of fruits. This highlights the importance of understanding the molecular mechanisms underlying fruit ripening across various species.

Previous studies on wild tomatoes and their relatives have categorized nine species into three groups based on their ripening behavior. These species produce climacteric ethylene, similar to cultivated tomato fruits that change color when fully ripened (Grumet et al., 1981; Chen, 2022). Climacteric fruits continue to ripen after harvest, and their shelf life is a decisive factor in the commercial lifespan of freshly harvested and stored tomatoes. Ripening-related genes, such as *PG*, *Psy*, and *ACO1*, play crucial roles in the biosynthesis of lycopene, ethylene, and cell wall degradation (Barry et al., 2000). Extending the shelf life of fruits is a significant breeding objective, and manipulating fruit ripening through transcriptional alterations in ripening-associated genes may contribute to achieving this goal (Causier et al., 2002).

During tomato fruit development, ethylene biosynthesis may be influenced differently at various stages and is proven to be related to fruit ripening and shelf-life duration. Based on this fact, the current study was performed to evaluate the ripening characteristics and shelf life of newly imported greenhouse tomato cultivars in Saudi Arabia by employing quantitative polymerase chain reaction (qPCR) analysis, provide valuable insights into the role of *ACS* and *ACO* in the production of ethylene, and support breeding efforts to enhance the shelf life of tomatoes.

Materials and Methods

Plant materials and growth conditions.

Three commercial tomato cultivar seeds provided from three different commercial sources (Roma: Red gold, Sakura F1: Enza Zaden, and GS12: SYNGENTA) were used in the current experiment. In a completely randomized design, the plants' seeds were cultivated in a rain-sheltered and non-heated greenhouse at the Department of Biology, College of Science, Jouf University (Sakaka, Saudi Arabia), from April 2021 to January 2022. Fertilization was applied once a week utilizing NPK (Polyhalite fertilizer, UK). Transplantation was applied using seedlings at 50 days old. The plants were cultivated with a single stem vertically and stopped employing cutting at the second leaf above the ninth truss. The tomato fruits set in the trusses had four or five fruits each. The tomato fruits utilized in this trial were collected at different stages: mature green (MG), breaker (BR), and full red (FR). Ripening stages were harvested and characterized based on the USDA color index (López Camelo & Gómez, 2004; examples for Roma and Sakura F1 are shown in Figure 1).

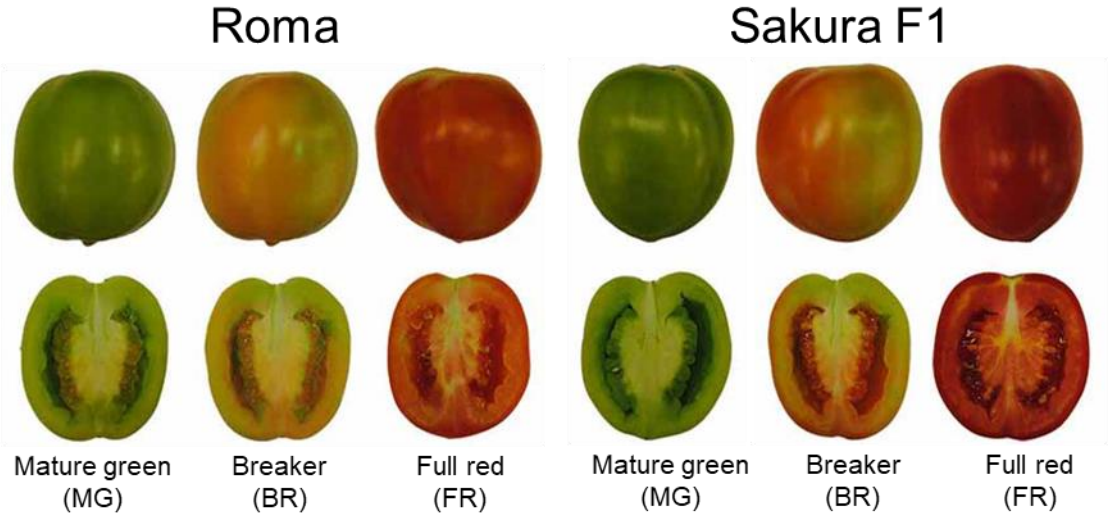

Fig. 1. Illustration of ripening stages of tomato fruits for Roma and Sakura F1 cultivars harvested at mature green (MG), breaker (BR), and full red (FR) stages defined according to the USDA color index.

RNA extraction and cDNA synthesis

The experiment was applied as outlined by the method presented by Kim et al. (2006), with a slight modification. Three biological replications from each sample were used, and total RNA was extracted from fresh fruit samples utilizing the Triazole® reagent (Sigma-Aldrich, MO, USA) and purified employing phenol-chloroform extraction. The quality of the RNA was checked through agarose gel electrophoresis using a 2 µg RNA loading buffer (FUJIFILM). RNA concentrations were determined using Quantus™ Fluorometer (Promega, USA). RNase-free DNase I (Promega, WI, USA) was employed to treat the isolated total RNA at 37 º C for 45 min. The purified RNA concentrations (1.5 and 2 µg) were utilized from the MG, BR, and FR stages, and the same order was employed for the synthesis of first-strand cDNA utilizing M-MLV® reverse transcriptase (Invitrogen, CA, USA), following the kit manual. When the process was implemented, cDNA was maintained at -20℃ up until quantification.

Real-time quantitative PCR (RT-PCR)

Real-time quantitative PCR was applied utilizing a 20 µl master mix which contains 0.5 µl primer 1 (P1), 0.5 µl primer 2 (P2) as listed in Table (1), 10 µl SYBR green (P3) in addition to 6.6 µl H2O, 1.2 µl buffer, and 1.2 µl of cDNA template along with a TaKaRa Taq polymerase (TaKaRa, Kyoto, Japan) kit. The qPCR cycle program was applied as follows: two min initial denaturation at 92 °C, denaturation phase at 92 °C/5 second, which was repeated for 40 cycles, annealing phase at 56 °C for 15 seconds, and extension phase at 72 °C for 26 seconds. A dissociation test was applied at 10-minute intervals from 95 °C to 50 °C to test for dimerization. The housekeeping gene glyceraldehyde- 3- phosphate dehydrogenase (*GAPDH*) gene was utilized as a reference gene. RT-PCRs were executed employing the RT-PCR platform Agilent Mx3000P.

Table 1. Oligonucleotide primers utilized in RT-PCR of ethylene-related genes.

Gene name	Gene product	Primer name	Sequence (5´ - 3´)
LeACSA	*ACS*	*LeACSAR*	GTCAAAATTATTGGAACTTAGTAGATG
		LeACSAF	CTAATTGAAAAGAACTACATGAAAAC
LeACOI	*ACO*	*LeACOIF*	AGGAAAAGCTACTTATAAGAATATGAA
		LeACOIR	GGTAATCTCTCCAATAAGAAGTAGTAG
LeGAPDH	*GAPDH*	*LeGAPDHF*	TGGTTAGATAAAATAGCTTAGAATAAA
		LeGAPDHR	AGATTACTAAACAAGGTTCAAAGTTAT

Statistical analysis

According to the Livak & Schmittgen (2001) method $2^{-\Delta\Delta CT}$, the cycle threshold (CT) was estimated according to the default setting of sequence detection of real-time software. To calculate the number of cDNA molecules, the equation on the graph per microgram from mRNA-converted cDNA was used. CT values were used to estimate gene expressions in terms of relative quantification. Utilizing the fold-change method, target genes were compared to the control. ANOVA One-Way analysis was conducted employing SAS v8.2 based on a general linear model together with Duncan's multiple range tests.

Results and Discussion

Real-time quantitative polymerase chain reaction (qPCR) has achieved widespread adoption as a gene quantitation method due to its numerous advantages. These include a large dynamic range, high sensitivity, sequence specificity, minimal post-amplification processing requirements, and the ability to increase sample throughput (Eum et al., 2009; Kitagawa et al., 2005).

In the present study, qPCR was utilized to assess the gene expression of *ACS* and *ACO* during various stages of tomato ripening, specifically the mature green (MG), breaker (BR), and fully ripe (FR) stages (Eum et al., 2009; Kitagawa et al., 2005). Suitable housekeeping controls were utilized to ensure accurate normalization of gene expression levels. These controls consisted of transcripts derived from various organs such as roots, flowers, fruits, and leaves. The selection of housekeeping controls aimed to identify at least one transcript that exhibited a two-fold range of expression across the organs analyzed (Alexander & Grierson, 2002; Kitagawa et al., 2005).

The housekeeping control gene glyceraldehyde- 3-phosphate dehydrogenase (*GAPDH*) was employed (Gachon et al., 2004; Wong & Medrano, 2005). *GAPDH* is widely used as a reference gene due to its relatively stable expression across various developmental stages and tissues (Gachon et al., 2004; Wong & Medrano, 2005; Yokotani et al., 2009; Sharma et al. 2021). By employing *GAPDH* as a housekeeping control, the gene expression levels of *ACS* and *ACO* could be accurately quantified in the context of tomato ripening stages, providing valuable insights into the regulation of

ethylene biosynthesis and fruit ripening processes. To ensure the successful and accurate quantification of gene amplification, a Melting Curve test was performed, demonstrating the presence of a single gene as indicated by one clear peak (Melting Curve test). Moreover, the target genes, *LeACSA* and *LeACO1*, were detected as single bands, affirming the primers' specificity for amplification (Melting Curve test; Table 2). The distinct peaks observed in the Melting Curve test confirmed the specificity of the amplification process and the absence of non-specific products.

Table 2. PCR cycle threshold obtained by RT-PCR for *GAPDH*, *LeACSA*, and *LeACO1* at MG, BR, and FR stages of the three tomato cultivars under study.

Tomato line ID	*GAPDH*	*LeACSA*			*LeACO1*		
	HKG	MG	BR	FR	MG	BR	FR
Roma	29	26	24	32	23	22	25
Sakura F1	29	29	27	28	28	29	33
GS12	29	28	22	31	24	25	29

The amplification curves generated in this study were instrumental in obtaining quantifiable values represented by the CT (cycle threshold) values. Each target gene, *LeACSA* and *LeACO1*, exhibited distinct amplification curves, reflecting the effectiveness and specificity of the primers used. CT values were extracted for all samples and their replicates, allowing for quantitative analysis of gene expression levels across different ripening stages and cultivars. Upon analyzing the CT values, it was observed that they ranged from 22 for the *LeACSA* gene in the BR stage of the GS12 cultivar to 33 for the *LeACO1* gene in the FR stage (Table 2). These variations in CT values across different genes and ripening stages provide insights into the differential expression patterns and the regulatory mechanisms associated with fruit ripening in the evaluated tomato cultivars.

To assess changes in gene expression levels, the ΔCT (delta CT) was calculated by subtracting the CT values of each target gene from the CT values of the housekeeping gene *GAPDH* (Livak & Schmittgen, 2001). This measure allowed for the estimation of relative expression levels and provided insights into the regulation of gene expression over time. The rate of change was calculated for each cultivar to identify the fold alteration in gene expression across different cultivars. In the case of the *LeACSA* gene, the maximum level of gene expression was observed in the BR stage for the GS12 cultivar, in contrast to the other two cultivars. On the other hand, all the cultivars exhibited relatively low expression levels of the *LeACSA* gene at the FR stage compared to the other two stages. The Sakura F1 showed a low level of *LeACSA* at the MG and FR stages compared to the BR stage, and lower than Roma and GS12 cultivars (Figure 2).

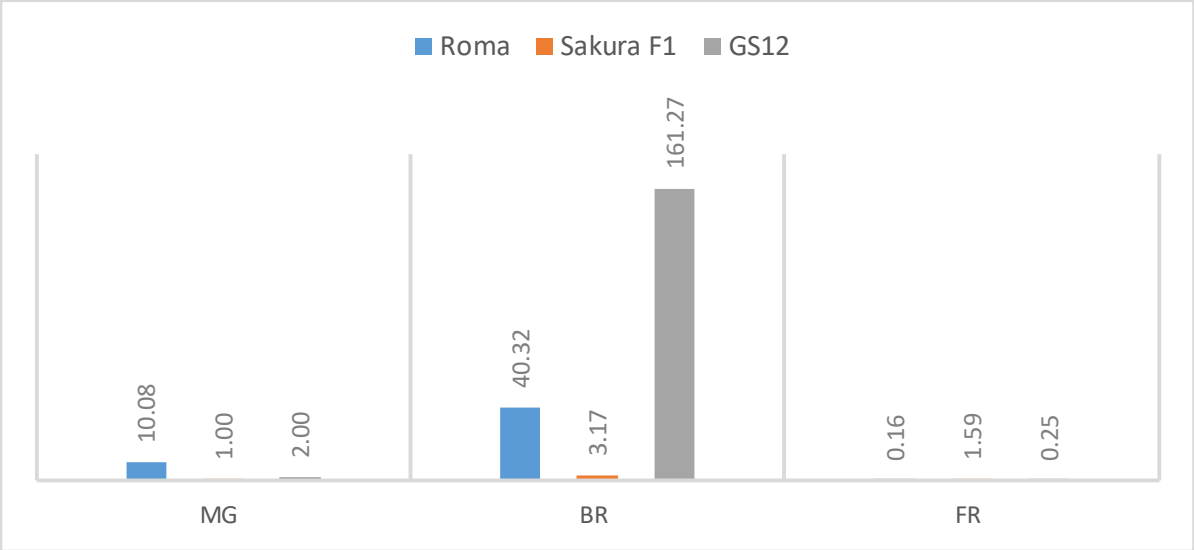

Fig. 2. The fold change ratios of relative expression for the two-ethylene synthase associated genes (*ACS*) measured at three fruit-growth stages for the three imported tomato cultivars.

The obtained data from all tested tomato cultivars indicate that the *ACS* gene, which is involved in ethylene production, exhibits distinct expression patterns corresponding to the stage of full red ripeness (FR). Notably, the *LeACSA* mRNA accumulates in climacteric fruits, as well as in ripening fruits treated with 1-methylcyclopropene, a compound known for hindering ethylene action in

several plant species (Blankenship & Dole 2003; Tatsuki et al. 2007). These findings suggest that the expression of *LeACSA* is tightly linked to the ripening process and is influenced by ethylene-signaling pathways, further underscoring its role in fruit ripening regulation. The genes *LeACS1*, *LeACS4*, and *LeACS2* play a crucial role in ethylene production during tomato fruit ripening (Hoogstrate et al. 2014). Transgenic tomatoes engineered to contain antisense *LeACS2* exhibit reduced ethylene production and fail to ripen, indicating the essential role of *LeACS2* and *LeACS4* genes in the ripening process (Oeller et al., 1991; Safhi et al. 2022). This knowledge provides valuable insights into ethylene synthesis and its impact on fruit ripening.

Interestingly, in the full red (FR) stage of the Sakura F1 cultivar, which demonstrated the lowest ethylene production during this stage, the expression of *LeACS1* was slightly decreased compared to the BR stage, while significantly decreased for the other two cultivars. This observation suggests that the downregulation of *LeACS1* may contribute to reduced ethylene production and potentially offer the opportunity for an extended shelf life in the Sakura F1 variety. Understanding the specific gene expression patterns and their relationship to ethylene production provides valuable information for developing tomato varieties with improved post-harvest characteristics (Yokotani et al., 2009; Sharma et al. 2021).

The *LeACO1* gene was overall lower than *LeACSA* and is expressed downgraded with fruit age among different stages, except for the Roma cultivar, which showed an increase of the gene during the BR stage. In the Sakura F1 cultivar, the gene equal to *LeACSA* was very low compared to the other two cultivars at all stages (Figure 3). In contrast, *ACO* expression was predominantly related to the breaker (BR) and mature green (MG) stages of fruit development. Across all tomato varieties, the expression patterns of the *ACO* gene were similar during these stages. However, in the Sakura F1 variety, the expression of the *LeACO1* gene exhibited an increase in the full red-ripe (FR) stage compared to the mature green (MG) stage (Zarembinski & Theologis, 1994; Wang et al. 2006).

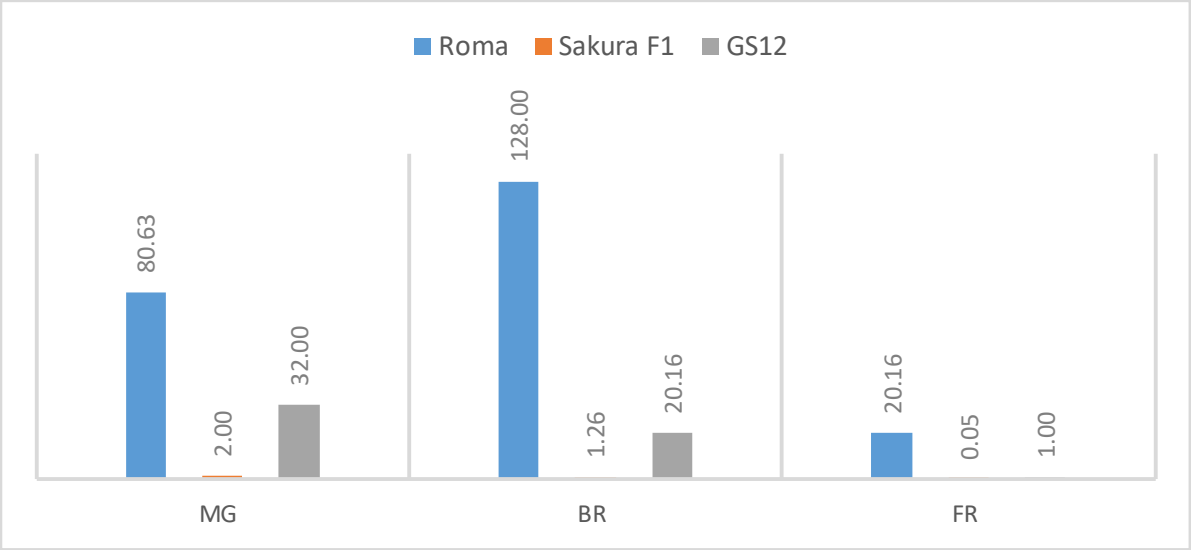

Fig. 3. The fold-change ratios of relative expression for the two-ethylene synthase associated genes (ACO1) measured at three fruit-growth stages for the three imported tomato cultivars.

This distinct expression pattern implies a prospective role for *LeACO1* in the final stages of fruit ripening in the Sakura F1 cultivar. Interestingly, the expression levels of the *ACO* gene (*LeACO1*) differed from those of the *ACS* genes, which displayed high similarity between the breaker (BR) and mature green (MG) stages when compared to the housekeeping gene *GAPDH*. This discrepancy suggests that the regulation of *LeACO1* through fruit ripening may differ from that of the *ACS* genes. Previous studies have hypothesized a negative regulation of *LeACO1* expression through fruit ripening of tomatoes, supporting the idea that *ACO* gene expression is under tight control during this process (Nakatsuka et al., 1998; Wang et al. 2006).

Previous studies have demonstrated a significant *ACO1* and *ACS* mRNA accumulation in completely red-ripe (FR) fruits. It has been reported that both transcriptional and posttranscriptional mechanisms contribute to regulating *ACS* activity. In comparison to the housekeeping gene *GAPDH*, the RT-PCR results revealed higher expression of *ACS* in the FR stage compared to the MG stage (Yang & Oetiker et al. 1994, Wang et al. 2006). In the context of the obtained data, inhibiting *ACS* activity in the fruit development system of the Sakura F1 cultivar caused a slight reduction in *LeACS1*

expression after ripening. This decrease in *LeACS1* expression may have implications for the prolonged ripening process and subsequently affect the duration of fruit firmness. Throughout the ripening process, *ACS* and *ACO* genes play a crucial role in regulating ethylene production, and they contribute to the progressive changes related to fruit ripening and the subsequent softening of the fruit (Barry et al. 2000; Barry & Giovannoni, 2007).

The current study highlights the low gene expression patterns related to ethylene production in the Sakura F1 cultivar, which can be utilized in breeding programs of tomatoes to realize core cultivars with an extended shelf-life duration. These findings have practical implications for improving tomatoes' post-harvest quality and commercial viability.

Conclusion

The findings of this study provide valuable insights into ethylene biosynthesis and its association with the fruit ripening progress in tomatoes, highlighting interspecific variations. The activation of *ACS* gene expression in fully red-ripe tomatoes was observed to significantly increase the expression of the *LeACS1* gene during the ripening process. Furthermore, it is proposed that the *ACS* and *ACO* genes play a crucial role in the levels of expression regulation and influencing the development of different fruit ripening processes. Notably, the low expression of the *LeACO1* gene was identified as a critical factor in suppressing ethylene production during the tomato fruit development stage (Wang et al. 2009). The *ACS* and *ACO* expression levels were determined using RT-PCR technology, with transcript measurements normalized using suitable housekeeping genes across different stages of tomato development in three distinct commercial cultivars. Through a comprehensive evaluation of candidate gene expression stabilities across samples from tomato plants, the results recommend the cultivar Sakura F1 for integration into tomato breeding programs alongside local tomato cultivars. This cultivar exhibited the most extended shelf-life duration, making it a promising candidate for enhancing the commercial viability and market longevity of tomatoes.

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