

Article

Stimulation of Absciscic Acid Biosynthesis by Ethylene with Suppressive Action of MiR396 in Postharvest Strawberry Fruits

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Abstract

Both abscisic acid (ABA) and ethylene play essential roles in the ripening process of strawberry fruit (*Fragaria × ananassa* Duch.). In this study, the crosstalk between ABA and ethylene involved in strawberry fruit ripening was investigated. The results showed that ethylene triggered a 1.30-fold increase in ABA levels by suppressing the expression of miR396. Meanwhile, the silencing of miR396 (STTM396 reporter) demonstrated that miR396 acts as a negative regulatory factor of ABA biosynthesis by repressing *FaNCED2* expression. Additionally, dual-luciferase assays indicated a 0.56-fold suppression of *FaNCED2* promoter activity by miR396. These findings reveal a novel regulatory mechanism in which ethylene promotes ABA biosynthesis through the suppression of miR396 expression in postharvest strawberry fruit.

Keywords: strawberry fruit ripening; abscisic acid biosynthesis; ethylene; MiR396; *FaNCED2*



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1. Introduction

The developmental progression of fruit ripening involves precisely orchestrated transcriptional reprogramming that governs discrete metabolic shifts, including anthocyanin accumulation, cell wall remodeling, and osmolyte biosynthesis [1–3]. Extensive studies in model non-climacteric species have elucidated unique regulatory networks distinct from ethylene-dependent pathways, particularly through advanced functional genomics and CRISPR-Cas9-mediated gene editing [4–7]. Recent multi-omics integration (transcriptome–proteome–metabolome) has further elucidated the spatiotemporal regulation of ripening-related phytohormones (ABA, IAA) and their crosstalk with epigenetic modifiers [8,9].

Recent advances in non-climacteric fruit ripening research had established abscisic acid (ABA) as the master regulator coordinating metabolic networks across species. Particularly in strawberry fruit (*Fragaria × ananassa*), ABA homeostasis orchestrated by *FaNCED1* enzymatic balance coordinated sucrose synthase induction. Meanwhile, the FaMYB10-TTG1 transcriptional complex formation and terpene synthase (FaTPS1) activation has created a synchronized ripening signature across sugar metabolism, pigmentation, and volatile profiles [10–14]. Moreover, emerging evidence had redefined the role of ethylene in

strawberry fruit ripening, indicating its function for non-climacteric species. Exogenous ethylene application induced sucrose synthase (FaSS) and cell wall-degrading enzymes such as pectin methylesterase (FaPME), thereby promoting fruit softening and sugar accumulation [15–17]. More importantly, a significant phytohormonal crosstalk existed between ethylene and ABA, where ethylene signaling synergistically up-regulated the expression of *FaNCED1*, rate-limiting genes in ABA biosynthesis, amplifying ABA accumulation, and establishing a hormonal relay that synchronizes pigmentation and textural changes [18,19].

The phytohormonal orchestration of strawberry fruit ripening has involved sophisticated ethylene-ABA cross-communication, where ethylene signaling initiated ABA biosynthesis through defined molecular cascades. In *Fragaria × ananassa* cv. ‘Sonata’ fruit, ethylene perception via FaETR1 receptors triggered CTR1 kinase-dependent phosphorylation cascades, activating the FaERF3-mediated transcriptional network that directly binds to the *FaNCED1* promoter, resulting in the up-regulation of this rate-limiting enzyme in ABA biosynthesis. Prolonged ethylene exposure induced spatial ABA dynamics accumulation, concurrent with temporal *FaNCED1* expression patterns. This hormonal synergy was amplified through a feedforward loop where ABA reciprocally enhanced ethylene sensitivity by increasing the stability of FaEIN3 transcription factors. The resultant metabolic reprogramming coordinates sucrose transporter activation, the epigenetic modification of anthocyanin genes, and miRNA-mediated cell wall remodeling, establishing an integrated regulatory architecture that synchronizes pigment biosynthesis, textural modification, and carbohydrate mobilization during ripening progression [20–22].

In the epigenetic regulation of strawberry fruit ripening, plant microRNAs (miRNAs, 20–22 nt non-coding RNAs) function as master switches, coordinating phytohormone signaling and metabolic transitions. In strawberry fruit, ripening-associated miRNA networks have exhibited functional divergence: the miR164-directed cleavage of FaNAC042 delayed senescence through pectate lyase suppression [23], while miR73 down-regulation activated polygalacturonase (FaPG) expression, accelerating cell wall disassembly [24]. Ethylene-responsive miRNA profiling across species reveals conserved regulatory nodes. Our previous work verified that ethylene suppressed miR161 expression to promote ABA biosynthesis through the activation of *FaNCED1* in strawberry fruit, underscoring the potential of miRNAs as mediators of ethylene-ABA crosstalk [25].

Building upon these foundations, our study deciphered the strawberry fruit miR396 regulatory axis targeting *FaNCED2*, a rate-limiting enzyme in ABA biosynthesis. In the present work, we aimed to verify an ethylene-ABA interplay through miR396 involved in the ripening of strawberry fruit. The function of miR396 as a responder of ethylene to inhibit ABA biosynthesis was demonstrated by exogenous ethephon and 1-methylcyclopropene (1-MCP). The regulatory function of miR396 on *FaNCED2* was demonstrated with short tandem target mimic (STTM) technology and dual-luciferase assays.

2. Materials and Methods

2.1. Fruit Material and Treatments

Mature strawberry (*Fragaria × ananassa* Duch. Cv. Akihime) fruits were harvested from a commercial plantation in Hangzhou, China (29°11′–30°33′). Flowers at anthesis were tagged for developmental stage monitoring. Fruits were categorized into six distinct developmental stages according to Lu’s method [26]: small green (SG, 7 days post anthesis, DPAs), large green (LG, 16 DPAs), white (W, 19 DPAs), initial red (IR, 22 DPAs), partially red (PR, 25 DPAs), and fully red (FR, 28 DPAs). A total of 390 white-stage (W) fruits were harvested, and 30 fruits of each other developmental stages were harvested for temporospatial analysis. Concurrently, roots, stems, leaves, and petals were collected from the same plants.

A total of 360 white-stage (W) fruits with a uniform size were selected and randomly divided into three treatment groups: (1) control (distilled water), (2) ethephon solution (eth), (3) 1-methylcyclopropene (1-MCP) according to Chen's method [23]. Fruits were immersed into 7 mM ethephon (Aladdin Industrial Inc., Shanghai, China) solution, 1×10^{-6} g L⁻¹ 1-MCP (Aladdin Industrial Inc., Shanghai, China) solution, and distilled water at room temperature for 5 min, respectively. Fruits were subjected to vacuum infiltration (0.05 MPa, 2 min) followed by air-drying at ambient temperature (25 ± 1 °C). All treated fruits were packaged in polyethylene (PE) pouches (H₂O vapor permeability: 5.51 g m⁻² at 20 °C) and stored in climate-controlled chambers (20 °C, 85% RH) for subsequent analyses after treatments [27]. Vegetative tissues (roots, stems, and leaves) and floral organs (petals) were immediately frozen in liquid nitrogen after harvest and stored at -80 °C for temporal expression profiling. Fruit samples for molecular analyses were collected at 24 h intervals, rapidly frozen in liquid nitrogen, and maintained at -80 °C until use [23].

2.2. Fruit Quality Measurements

Ten randomly selected fruits per treatment group were subjected to non-destructive quality assessments. All measurements were conducted at symmetrical equatorial positions (two sites per fruit) to ensure data representativeness. Fruit surface color was quantified using a Chroma Meter CR-400 (Konica Minolta Sensing, Osaka, Japan). The *a** value was recorded according to CIELAB color space specifications. Fruit firmness was determined using a TA-XT2i texture analyzer (Stable Micro Systems, Godalming, UK) equipped with a 2 mm cylindrical probe [28]. Total anthocyanin content (TAC) was determined through the pH differential method [29].

2.3. Ethylene Production

Ethylene production was quantified using a gas chromatography-based approach according to Chen et al. [25]. A sample of ten strawberries was enclosed in a 2 L airtight vessel and maintained at 20 °C for 2 h to facilitate ethylene accumulation. Following incubation, a 2 mL aliquot of headspace gas was sampled and injected into a gas chromatograph (GC) equipped with a flame ionization detector (FID) (SHIMADZU, Kyoto, Japan). Separation was achieved using a 2 m × 3 mm aluminum oxide column held at a constant temperature of 85 °C. The ethylene concentration was determined by calibrating the peak areas against a certified ethylene standard gas at a concentration of 1×10^{-5} L L⁻¹.

2.4. ABA Extraction and Analysis

Approximately 4.0 g of fruit sample was homogenized with 0.01 L ultrapure water and centrifugation (4 °C, $10,000 \times g$ for 30 min) was carried out subsequently. The supernatant was adjusted to pH 2.8 using 15% acetic acid, and then the mixture was extracted twice with an equal volume of diethylether. The organic portion was collected and evaporated softly under nitrogen flow. The residue was resolved in 5×10^{-4} L 80% (*v/v*) methanol solution. The quantification of abscisic acid (ABA) was performed by ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS). Separation was carried out on a Waters Acquity UPLC HSS C18 column (100 mm × 2.1 mm, 1.8 µm) with the column oven set to 40 °C. The mobile phase consisted of 0.01% acetic acid in water (A) and 0.01% acetic acid in acetonitrile (B), delivered at a flow rate of 0.50 mL min⁻¹ under the following gradient: initial 95% A, decreased to 60% A over 1 min, then to 5% A over 6 min, held for 2 min, and returned to 95% A in 1 min. The effluent was monitored by a Xevo G2S q-TOF mass spectrometer operating in ESI-positive mode. Key MS parameters included a capillary voltage of 2.5 kV, desolvation gas (N₂) flow of 900 L h⁻¹ at 500 °C, and source temperature of 150 °C. The identification and quantification

of ABA were confirmed by matching the retention time and exact mass to those of an external standard, processed using TargetLynx Application Manager (Agilent Technologies Inc., Santa Clara, CA, USA) [30,31].

2.5. RNA Extraction, cDNA Synthesis, and RT-qPCR

Total RNA was extracted using a CTAB-based protocol [32]. Briefly, 1.0 g of powdered sample was homogenized in 4 mL CTAB extraction buffer (2% CTAB (Aladdin Industrial Inc., Shanghai, China), 100 mM Tris-HCl pH 8.0 (Aladdin Industrial Inc., Shanghai, China), 25 mM EDTA (Aladdin Industrial Inc., Shanghai, China), 2 M NaCl (Aladdin Industrial Inc., Shanghai, China)) containing 2% β -mercaptoethanol, followed by 65 °C incubation with intermittent vortexing. After phase separation via chloroform/isoamyl alcohol (24:1) extraction and LiCl (Aladdin Industrial Inc., Shanghai, China) precipitation, RNA pellets were dissolved in pre-warmed SSE buffer, subjected to phenol/chloroform purification, and ethanol-precipitated. RNA integrity was verified spectrophotometrically ($A_{260}/A_{280} = 1.8\text{--}2.1$) using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Genomic DNA was eliminated from 1 μ g total RNA using gDNA Eraser (RR071A, Takara Bio Inc., Osaka, Japan) at 42 °C for 5 min, followed by first-strand cDNA synthesis with Oligo dT/Random Hexamers in a 20 μ L reaction (42 °C, 15 min; 85 °C, 5 s). Synthesized cDNA was stored at -80 °C until qPCR analysis. Genomic DNA elimination and first-strand cDNA synthesis were performed using PrimeScript™ RT Reagent Kit with gDNA Eraser (RR071A, Takara Bio Inc., Osaka, Japan) following the manufacturer's protocol.

Gene expression quantification was performed using ChamQ™ SYBR Color qPCR Master Mix (Q311, Vazyme Biotech Co., Ltd., Nanjing, China) on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Amplifications were carried out in 20 μ L reactions containing 2 μ L cDNA template (diluted 1:10) with the following thermal profile: 95 °C for 30 s; 40 cycles of 95 °C for 5 s; and 60 °C for 30 s. Specific primers for *FaActin* (GenBank: AB116565) and *U6* snRNA (MK976922) were employed as endogenous controls for mRNA and miRNA normalization, respectively. Melt curve analysis (60–95 °C, 0.3 °C increment) confirmed the amplification specificity. Relative expression levels were calculated via the $2^{-\Delta\Delta C_t}$ method with three technical replicates per biological sample.

2.6. Plasmid Construction and Agro-Infiltration

Tobacco rattle virus (pTRV1 and pTRV2) VIGS vectors were selected to silence miR396 in this study. The full length of STTM396 (Table S1) was transferred into the BamHI and EcoRI restriction sites of the pTRV2 vector. pTRV1, pTRV2, and pTRV2-STTM396 vectors were transferred into *Agrobacterium tumefaciens* (EHA105) (WEIDI Bio Inc., Shanghai, China). *A. tumefaciens* were grown at 28 °C in YEB liquid medium. The OD_{600} of *A. tumefaciens* cultures was adjusted to 1.0, and 1×10^{-3} L of the suspension was injected into the stalk of strawberry fruits at the W stage [33].

2.7. Dual-Luciferase Reporter

To elucidate the regulatory effects of miR396 on the transcription level of *FaNCED2*, we engineered a dual-luciferase reporter [34]. Full lengths of Ade-MIR396 were constructed into the pGreen II 0029 62-SK (SK) vector (WEIDI Bio Inc., Shanghai, China). The target site of miR396 in *FaNCED2* was inserted into the EcoRI and NotI restriction sites of the 3'-UTR sensor vector (Addgene, accession number 55206), and the full sequence of the *FaNCED2* promoter was constructed into the HindIII and BamHI restriction sites of the pGreen II 0800-LUC (LUC) vector (WEIDI Bio Inc., Shanghai, China). Meanwhile, the perfect target sites of miR396 and 21-nt spacer (Table S1) were transferred into the same restriction sites

of the 3'-UTR vector as a positive and negative control, respectively. All plasmids were transferred into *Agrobacterium tumefaciens* (EHA105 pSoup) (WEIDI Bio Inc., Shanghai, China). All *A. tumefaciens* were infiltrated into *Nicotiana benthamiana* leaves. Three days later, the firefly luciferase (FLUC) and the Renilla luciferase (RLUC) of *N. benthamiana* leaves were measured with a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

2.8. Statistical Analysis

Quantitative results derived from triplicate experimental iterations are expressed as mean $\pm$ SD ($n = 3$). All of the results were obtained from three biological replicates by using a randomized block design. The experimental design necessitated the implementation of a factorial ANOVA with post hoc evaluation using Duncan's multiple range test, executed through SPSS Statistics (IBM Corp., Version 26.0, Armonk, NY, USA). Pearson's correlation analysis was carried out using the R Programming Language (Rgui, version 64 4.1.10).

3. Results

3.1. Temporospatial Expression of miR396

To investigate the expression pattern of miR396 in strawberry, stem-loop primers were specifically designed for reverse transcription followed by quantitative real-time PCR (qRT-PCR) analysis across various strawberry tissues and distinct fruit ripening stages. As shown in Figure 1A, miR396 exhibited tissue-specific expression with predominant accumulation in the leaves and fruits, while no detectable expression was observed in the roots or stems. Fruit developmental stages were categorized based on days post anthesis (DPAs): small green (SG, 7 DPAs), large green (LG, 16 DPAs), white (W, 19 DPAs), initial red (IR, 22 DPAs), partially red (PR, 25 DPAs), and fully red (FR, 28 DPAs). Notably, miR396 transcript levels demonstrated a progressive decrease throughout fruit maturation (Figure 1B). This sustained down-regulation pattern suggests a negative correlation between miR396 expression and strawberry fruit ripening progression.

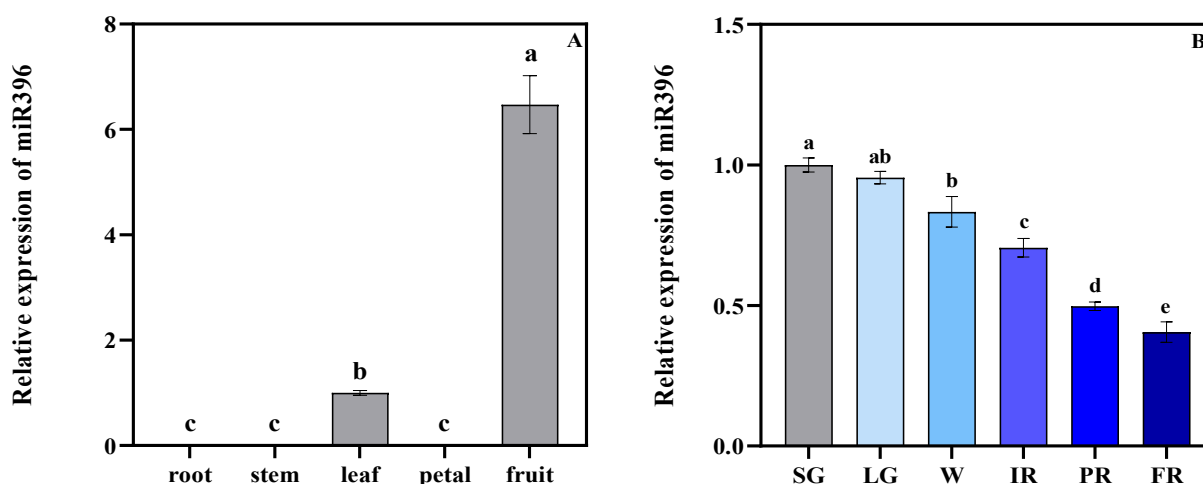


Figure 1. Temporospatial pattern analysis of miR396 expressions in strawberry plant. (A) Different tissues of strawberry plant; (B) strawberry fruit was divided into 6 maturity stages—SG (small green); LG (large green); W (white); IR (initial red); PR (partial red); and FR (full red)—at 7, 16, 19, 22, 25, and 28 days after anthesis. Error bars indicate the standard deviation (SD) of three replications and different letters (a–e) indicate significant differences at $p < 0.05$.

3.2. Ethylene Promoted Anthocyanin Accumulation and Softening of Strawberry Fruit

As depicted in Figure 2A, no visual alterations in the strawberry fruits' external appearance were observed at day 2 post treatment. By day 4, exogenous ethephon treatment

accelerated red coloration compared with the control group, whereas 1-MCP treatment mildly retarded this pigmentation process. The quantitative assessment of the a^* value revealed that ethephon-treated fruits exhibited a 33.4% increase, while 1-MCP-treated samples showed a 29.7% reduction relative to controls at day 4 (Figure 2B).

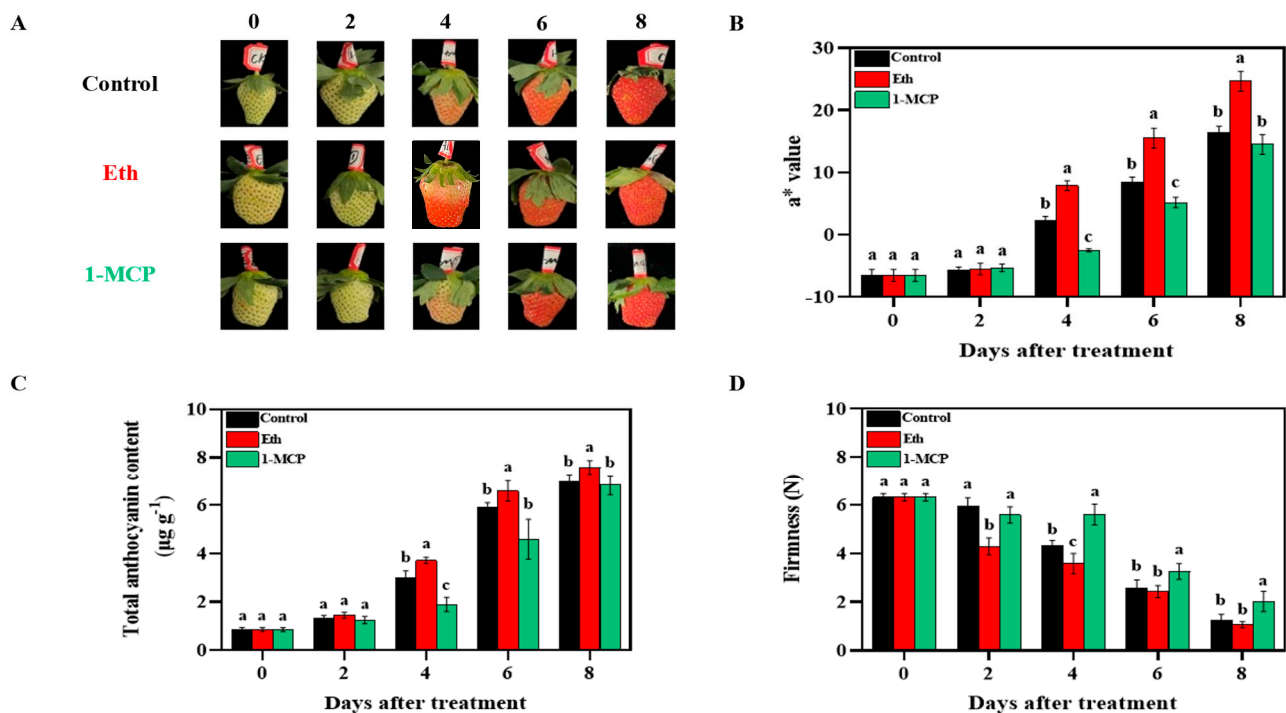


Figure 2. Influence of ethephon and 1-MCP treatments on appearance (A), coloration (B), total anthocyanin content (C), and firmness (D) of strawberry fruit. Error bars indicate the standard deviation (SD) of three replications and different letters (a–c) indicate significant differences at $p < 0.05$.

Consistent with the phenotypic observations, significant divergence in total anthocyanin content, a pivotal determinant of red pigmentation, emerged among treatments at day 4. Ethephon application markedly enhanced anthocyanin biosynthesis (1.3-fold increase vs. control), contrasting with the suppressive effect (30% decrease) induced by 1-MCP (Figure 2C). Fruit firmness, another critical maturity index, displayed treatment-specific dynamics. The ethephon group exhibited significantly lower firmness than other treatments by day 2. By day 4 post treatment, paradoxical firmness patterns emerged: fruits treated with 1-MCP paradoxically demonstrated 22.5% higher firmness than controls, whereas ethephon application resulted in 12.3% softening (Figure 2D).

3.3. Change in *Mir396*, and *FaNCED2* Expression and ABA Content After Treatments

To elucidate the ethylene-mediated regulation of *miR396* expression, a stem-loop primer-based qRT-PCR was conducted on strawberry fruits subjected to ethephon and 1-MCP treatments. The *miR396* abundance displayed a progressive attenuation during post-treatment storage. Compared with the control group, ethephon treatment induced a significant down-regulation of *miR396* (0.67-fold relative expression), whereas 1-MCP treatment effectively mitigated this suppression (1.33-fold relative expression) at day 4 post treatment (Figure 3A).

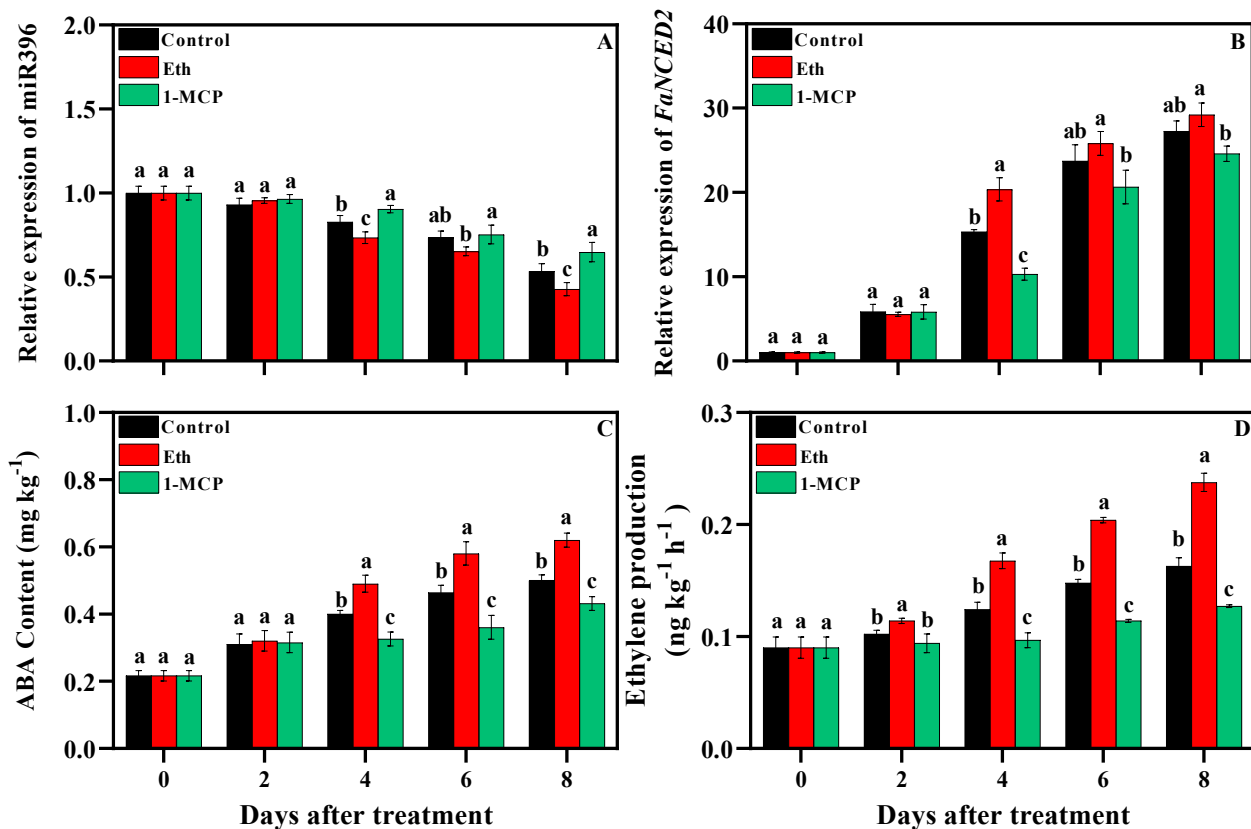


Figure 3. Influence of ethephon and 1-MCP treatments on relative expression of miR396 (A), *FaNCED2* (B), ABA content (C), and ethylene production (D) of strawberry fruit. Error bars indicate the standard deviation (SD) of three replications and different letters indicate significant differences at $p < 0.05$.

Intriguingly, an inverse regulatory pattern was observed in the expression of *FaNCED2*, a key ABA biosynthetic gene, whose transcript levels showed a time-dependent elevation throughout storage, with ethephon treatment inducing an upsurge while 1-MCP application reduced its expression (Figure 3B). Furthermore, ABA content exhibited a similar pattern. Ethephon treatment triggered a continuous accumulation of ABA, reaching 1.21-fold of control levels by day 8. Conversely, 1-MCP treatment attenuated this increase, maintaining 0.78-fold compared with controls at the same time point (Figure 3C). Similarly, ethephon treatment significantly promoted ethylene production from strawberry fruits from day 4 after treatment while 1-MCP treatment suppressed ethylene release (Figure 3D).

3.4. Correlation Analysis

To delineate the interrelationships among miR396, *FaNCED2*, ABA content, and strawberry fruit ripening, Pearson's correlation analysis was systematically performed (Figure 4). Strikingly, miR396 expression exhibited a robust negative correlation with *FaNCED2* transcript levels and ABA content, whose correlation coefficients reached -0.95 and -0.91 , respectively. Notably, the a^* value and total anthocyanin content (TAC) were inversely correlated with miR396 abundance ($r = -0.98$ and $r = -0.96$), whereas fruit firmness demonstrated a positive correlation with miR396 levels ($r = 0.95$). These multivariate correlation patterns collectively establish that miR396 functions as a negative regulator in strawberry fruit ripening through the coordinated modulation of pigment biosynthesis and cell wall metabolism.

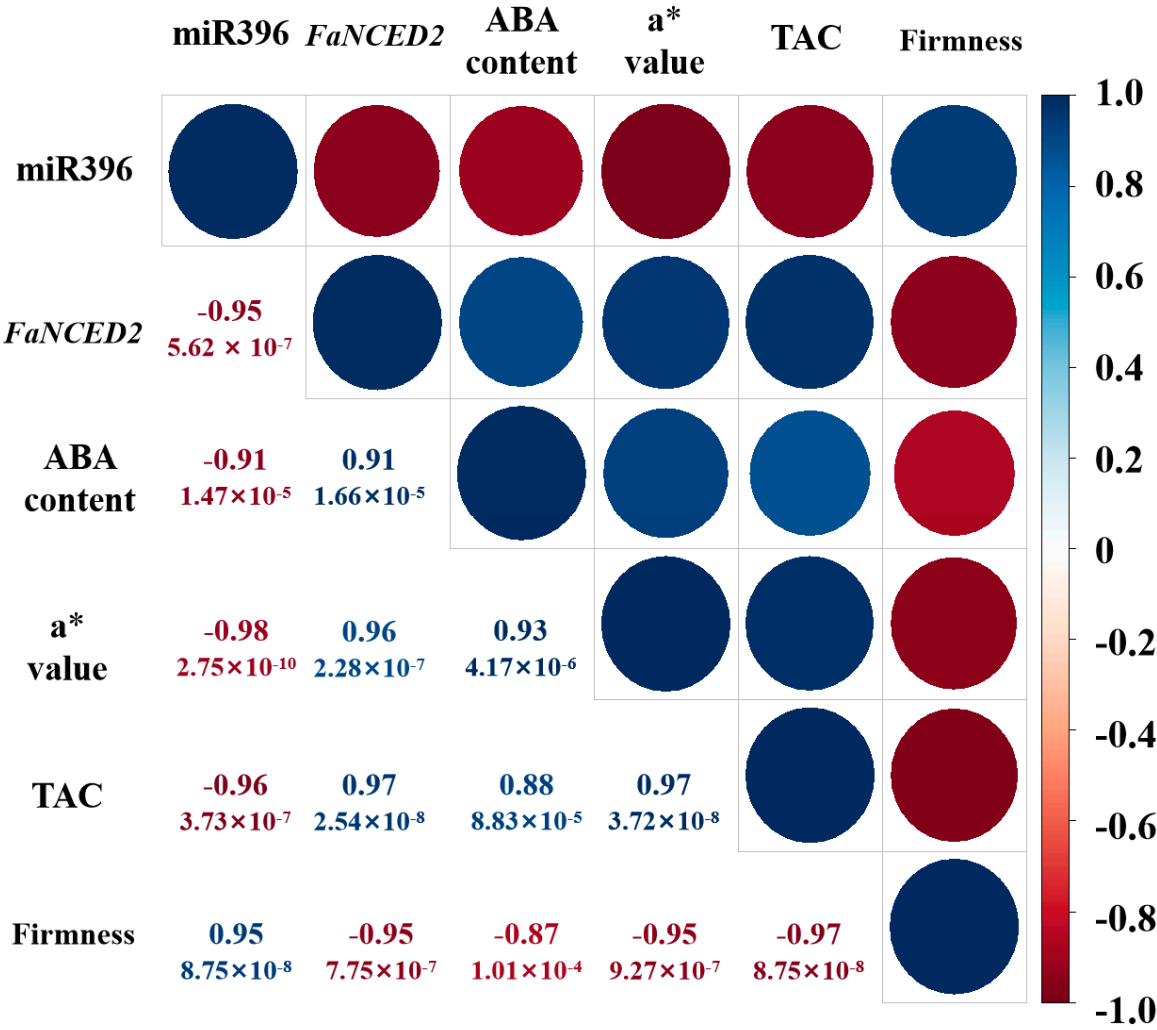


Figure 4. Correlation analysis between miR396 expression, *FaNCED2* expression, ABA content, *a** value of fruit, total anthocyanin content, and fruit firmness ($n = 39$). The blue and red colors represent positive and negative correlations, respectively. The counts represent the correlation coefficients (r) and the p -value, respectively.

3.5. Silencing of MiR396

To functionally characterize miR396 in strawberry ripening, a virus-induced gene silencing (VIGS) system was employed by co-infiltrating pTRV1 with either empty pTRV2 or recombinant pTRV2-STTM396 into white-stage (W) strawberry fruits. Phenotypic analysis revealed accelerated reddening in STTM396-infiltrated fruits compared with vector controls at day 5 post treatment (Figure 5A). qRT-PCR validation confirmed successful miR396 silencing, with STTM396 fruits exhibiting only a 0.41-fold compared with control fruits (Figure 5B). This microRNA silencing triggered the reciprocal regulation of downstream targets: *FaNCED2* expression surged 5.72-fold, and ABA content increased to 1.33-fold, correspondingly (Figure 5C,D). Concomitantly, colorimetric analysis demonstrated a 20% decrease in *a** value and 0.77-fold in total anthocyanins content in STTM396 fruits compared with controls (Figure 5E,F). Texture profile analysis further revealed a 1.92-fold change of firmness in miR396-silenced fruits compared with control fruits, corroborating the accelerated ripening phenotype (Figure 5G).

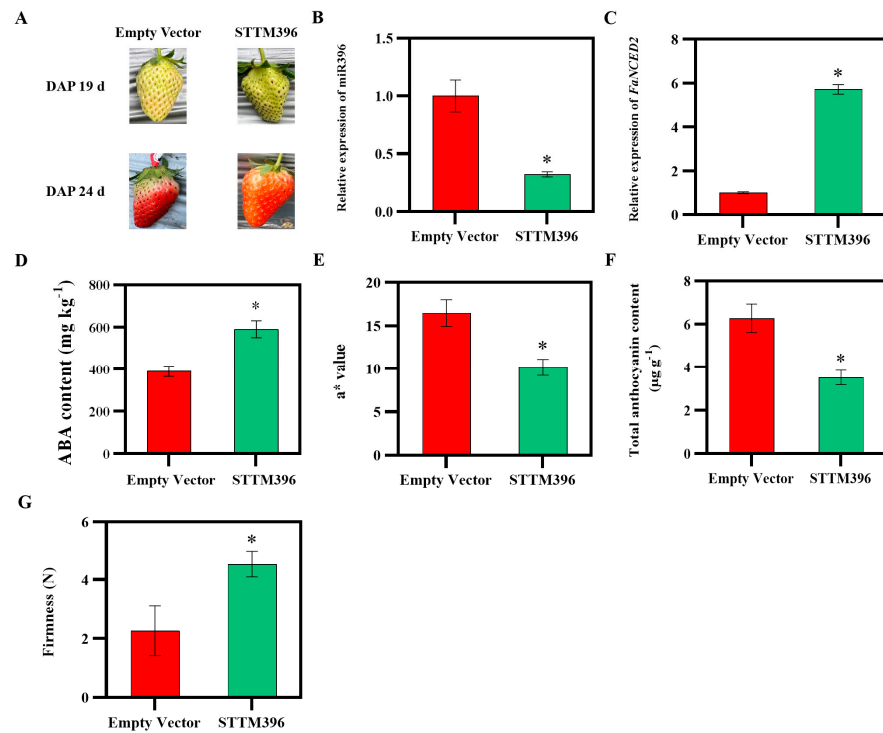


Figure 5. Influence of miR396 silencing on strawberry fruit ripening. (A) Fruit appearance; (B) relative expression of miR396; (C) relative expression of *FaNCED2*; (D) ABA content; (E) color of fruit; (F) total anthocyanin content; (G) fruit firmness. Error bars indicate the standard deviation (SD) of three replications and asterisks (*) indicate significant differences at $p < 0.05$.

3.6. Dual-Luciferase Reporter of MiR396 and *FaNCED2*

To mechanistically validate the miR396-mediated regulation of *FaNCED2*, dual-luciferase reporter assays were performed in *Nicotiana benthamiana* leaf epidermis. The *FaNCED2* promoter-driven LUC/REN ratio was significantly reduced to 50% of empty vector controls upon miR396 co-expression (Figure 6). This suppression effect was corroborated by positive controls containing perfectly matched target sites, whereas negative controls with scrambled 21-nt sequences showed no significant alteration. These findings demonstrated that miR396 directly repressed *FaNCED2* transcriptional activity through promoter binding, establishing a molecular framework where miR396 modulated strawberry ripening via ABA biosynthesis regulation.

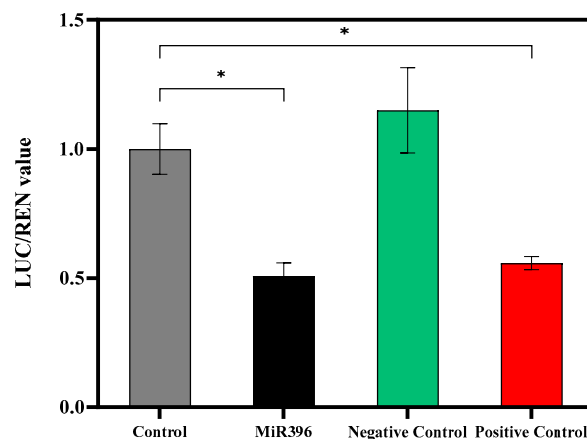


Figure 6. Dual-luciferase assays of the negative regulation of miR396 on the promoter of *FaNCED2*. The LUC/REN value of empty vector was set as 1. Error bars indicate the standard deviation (SD) of six replications and asterisks (*) indicate significant differences at $p < 0.05$.

4. Discussion

Emerging evidence has demonstrated the pivotal regulatory role of abscisic acid (ABA) in modulating strawberry fruit ripening processes, particularly through accelerating textural softening, anthocyanin-mediated chromatic transition, and phenolic compound metabolism [35–37]. Contemporary studies have elucidated a phytohormonal crosstalk wherein ethylene served as an upstream modulator of ABA metabolic flux. Notably, prolonged ethylene exposure was shown to elevate endogenous ABA concentrations in ‘Elsanta’ strawberries [21], with parallel findings in the ‘Sonata’ cultivar demonstrating ethylene-induced ABA accumulation patterns [22]. Our experimental data revealed that ethephon treatment triggered a 1.3-fold elevation in ABA levels (Figure 3C) concomitant with decreased firmness and enhanced anthocyanin biosynthesis (Figure 2C,D), which indicated the ethylene-mediated ABA synthesis increased ripening progression.

The epigenetic regulation of ripening has gained prominence with the discovery of miRNA-ethylene signaling interplay [38]. As post-transcriptional modulators, miRNAs typically executed gene silencing through either mRNA degradation or translational suppression (partial pairing) [39]. In ethylene perception cascades, the signal is initially captured by a five-member receptor family (ETR1, ERS1, ETR2, EIN4, and ERS2) that physically associates with CTR1 kinase. This interaction activates EIN2-mediated signal transduction, ultimately modulating downstream ethylene-responsive miRNAs [40–42]. Our transcriptomic profiling identified the significant down-regulation of miR396 in ethylene-treated strawberry receptacles. This suppression triggered the up-regulation of *FaNCED2* (1-aminocyclopropane-1-carboxylate oxidase), a rate-limiting enzyme in ABA biosynthesis. The direct interaction between miR396 and the *FaNCED2* promoter was experimentally verified using a dual-luciferase reporter assay in a heterologous *Nicotiana benthamiana* transient expression system. This platform was selected for its well-documented utility in delineating direct transcriptional and post-transcriptional regulatory mechanisms, particularly owing to its minimal endogenous background interference. Specifically, *N. benthamiana* exhibited low basal levels of miR396, and its native miRNA sequences were phylogenetically distinct from those of strawberry, thereby providing a reconstitution environment suitable for assessing the specific regulatory effect of strawberry-derived miR396 on its cognate promoter. The significant suppression of luciferase activity observed in our assays provided compelling evidence for a direct molecular interaction between miR396 and the *FaNCED2* promoter. While heterologous systems might not fully recapitulate the nuanced spatiotemporal dynamics or absolute strength of promoter activity as seen in the native strawberry fruit context, they offered a validated and controlled approach for establishing fundamental targeting relationships. The primary objective of this experiment was to test the hypothesis of direct targeting, a question for which the *N. benthamiana* system is particularly well suited [43–45]. Future studies employing genetic approaches to modulate miR396 levels directly in strawberry will be invaluable for elucidating the comprehensive physiological impact of this regulatory node on ABA biosynthesis and fruit ripening, which deserves further investigation.

Building upon our previous finding that ethylene suppressed miR161 to regulate *FaNCED1* expression [25], the present study identified a parallel regulatory circuit wherein the ethylene-mediated suppression of miR396 led to the up-regulation of *FaNCED2*. The discovery of this novel ethylene-miR396-*FaNCED2* module was significant, as it demonstrated that ethylene did not rely on a single pathway but rather orchestrated ABA biosynthesis through multiple, distinct miRNA-mediated circuits. This revealed a higher-order regulatory architecture for phytohormone crosstalk in strawberry fruit ripening. The co-existence of these two pathways (ethylene-miR161-*FaNCED1* and ethylene-miR396-*FaNCED2*) suggested a sophisticated mechanism for the fine-tuning of ABA accumulation. A key question

emerging from this model concerns the functional specialization of these parallel circuits. We postulated that they may operate with distinct spatiotemporal dynamics or respond to different internal or environmental cues, thereby providing the plant with robust and flexible control over the ripening process [46–48]. Future investigations are warranted to decipher the potential division of labor and synergistic relationships between these modules, which will be crucial for a systems-level understanding of hormonal regulation in non-climacteric fruits. In summary, these findings collectively support the prospective model (Figure 7) of ethylene-ABA crosstalk governing ripening dynamics.

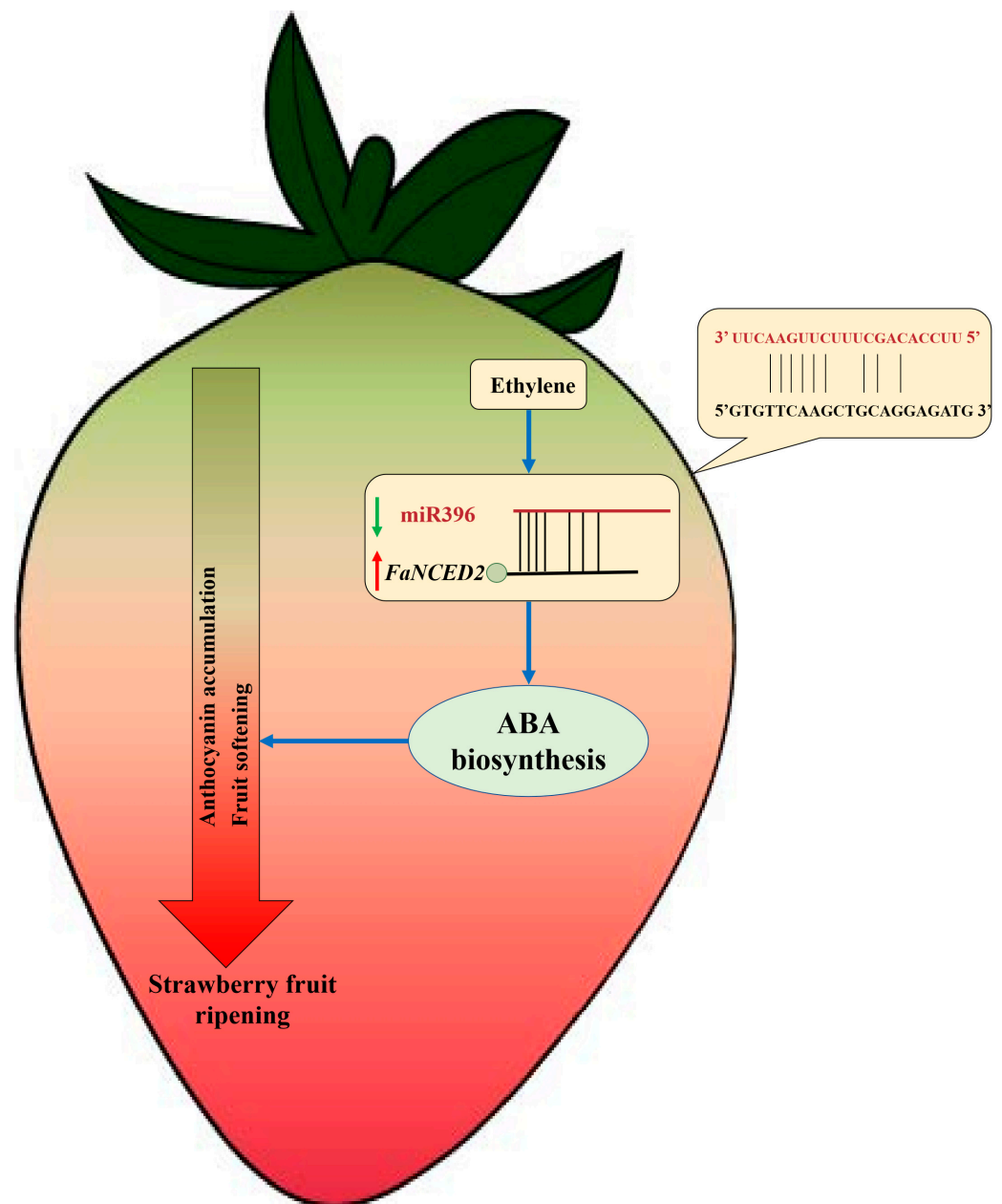


Figure 7. Schematic for the miR396 responding to ethylene signaling in the regulation of strawberry fruit ripening.

5. Conclusions

This investigation provides evidence that ABA biosynthesis in postharvest strawberry fruits was promoted by ethylene through the transcriptional regulation of miR396. During fruit ripening, the enhanced ABA content showed a positive correlation with exogenous

ethephon treatment. Molecular analyses revealed an inverse relationship between miR396 expression and ABA metabolic activity, where ethylene down-regulated miR396 transcription, concurrently alleviating its suppressive effects on ABA biosynthesis gene *FaNCED2*. These findings elucidate a previously uncharacterized regulatory network governing the hormonal crosstalk between ABA and ethylene during postharvest physiological processes in strawberry fruit.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae11111280/s1>, Table S1: Primers sequences used for qRT-PCR expression analysis in this study.

Author Contributions: R.C. and Y.Y. conceived and designed the experiments. R.C. performed the experiments, analyzed the data, and wrote the manuscript. W.G. and Z.H. also contributed to the data interpretation and writing. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data is contained within the article or Supplementary Material.

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Conflicts of Interest: The authors declare no conflicts of interest.

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