Hydrogen Sulfide Inhibits Fruit Softening by Regulating Ethylene Synthesis and Signaling Pathway in Tomato (*Solanum lycopersicum*)

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Abstract. Hydrogen sulfide (H_2S) has been proven to be a multifunctional signaling molecule in plants. In this study, we attempted to explore the effects of H_2S on the climacteric fruit tomato during postharvest storage. H_2S fumigation for 1 d was found to delay the peel color transition from green to red and decreased fruit firmness induced by ethylene. Further investigation showed that H_2S fumigation downregulated the activities and gene expressions of cell wall-degrading enzymes pectin lyase (PL), polygalacturonase (PG), and cellulase. Furthermore, H_2S fumigation downregulated the expression of ethylene biosynthesis genes *SIACS2* and *SIACS3*. Ethylene treatment for 1 d was found to induce the expression of *SIACO1*, *SIACO3*, and *SIACO4* genes, whereas the increase was significantly inhibited by H_2S combined with ethylene. Furthermore, H_2S decreased the transcript accumulation of ethylene receptor genes *SIETR5* and *SIETR6* and ethylene transcription factors *SICRF2* and *SIERF2*. The correlation analysis suggested that the fruit firmness was negatively correlated with ethylene biosynthesis and signaling pathway. The current study showed that exogenous H_2S could inhibit the synthesis of endogenous ethylene and regulate ethylene signal transduction, thereby delaying fruit softening and the ripening process of tomato fruit during postharvest storage.

Tomato (*Solanum lycopersicum* L.) is an important fruit for human consumption because it provides nutrients like flavor compounds, vitamins, and fiber (Beecher, 1998). Postharvest ripening and senescence involve changes in metabolism and gene expression, which further affect sensory attributes, flesh texture, fruit nutritional quality, and market acceptance. Fruit ripening and senescence are accompanied by texture softening, which is one of the most important factors regarding fruit quality and consumer acceptability (Giovannoni, 2007; Grierson et al., 1986; Musse et al., 2009). Fruit softening is a complex process that results from loosening

of the cell wall. Many enzymes such as pectinmethylesterase, polygalacturonase, cellulase, and pectin lyase catalyze the modification and degradation of cell wall components, including pectin, hemicellulose, and others (Fischer and Bennett, 1991; Gwanpua et al., 2016; Thompson et al., 1998). Therefore, delaying fruit softening will maintain the texture of fruit and improve the market values.

Ethylene is an important phytohormone in plants and is involved in many aspects of the plant life, including seed germination, root hair development, flower senescence, and fruit ripening (Sisler and Yang, 1984). To-

mato, as a typical respiratory climacteric fruit, ripening and senescence are largely dependent on endogenous production and ethylene action (Liu et al., 2015). Ethylene is generated from the precursor of methionine through Yang's cycle. Studies of ethylene biosynthesis have been focused on the isolation and characterization of *ACS* (1-aminocyclopropane-1-carboxylate synthase) and *ACO* (1-aminocyclopropane-1-carboxylate oxidase) genes, and *ACC* (1-aminocyclopropane-1-carboxylate) is the immediate precursor of ethylene (Adams and Yang, 1979; Alexander and Grierson, 2002; Wang et al., 2002). After synthesis, ethylene is perceived by ethylene receptors (ETRs) (Payton et al., 1996). Subsequently, a signaling cascade including both positive and negative regulators modulates a large family of transcription factors called ethylene response factors (ERF) (Thirugnanasambantham et al., 2015). Enormous studies demonstrated that the transcription factor ERF in the AP2/ERF family modulates metabolic pathways involved in fruit ripening and senescence, chlorophyll degradation, fruit softening, and changes in aromas (De Boer et al., 2011; Licausi et al., 2013; Qi et al., 2011). Because ethylene is the key phytohormone regulating fruit ripening, many studies have focused on delaying the action or synthesis of ethylene. For instance, ethylene antagonist 1-methylcyclopropene (1-MCP) was broadly applied to delay fruit ripening and senescence (Song et al., 2018; Watkins et al., 2000). Nitric oxide (NO), an important signaling molecule, was found to delay fruit softening in mango fruit during storage by inhibiting ethylene biosynthesis (Zaharah and Singh, 2011).

Hydrogen sulfide (H_2S) is found to be the third most important gasotransmitter in animals and plants after NO and carbon monoxide (CO) (Gadalla and Snyder, 2010; Lisjak et al., 2010; Wang, 2010). H_2S participates in almost all aspects of plant life, including seed germination, root organogenesis, stomatal movement, resistance to stress conditions, and plant senescence (Chen et al., 2011; Qiao et al., 2015; Wang et al., 2012; Zhang et al., 2009). Similar to the role of NO in the regulation of fruit ripening and senescence, accumulating studies found that H_2S could alleviate postharvest ripening and senescence by modulation of the antioxidant system of kiwifruit, apple, banana, and others (Gao et al., 2013; Ge et al., 2017; Hu et al., 2012). Whether and how H_2S is involved in the ripening and senescence of tomato are still unclear. Therefore, in this study, we attempted to illuminate the mechanism of H_2S in modulating tomato ripening and senescence. Additionally, the impact of H_2S on the ethylene synthesis and signaling pathway in tomato fruit ripening was investigated.

Materials and Methods

Plant materials and sample preparation. Tomatoes (*Solanum lycopersicum* cv. Micro Tom) were harvested at the mature green stage; plants were grown in the greenhouse at Hefei University of Technology in Hefei, China. The tomato plants were cultivated at

24 °C with 16-h day and 8-h night conditions, with relative humidity at 50% to 65%. The media for culturing tomato seedlings was composed of nutritional soil, peat soil, and vermiculite in a ratio of 3:5:1. Thirty days post anthesis tomato fruits, which is in the mature-green stage, with similar size and without physical injuries or infections were chosen and stored in containers. The tomatoes were fumigated with ethylene (C_2H_4) released from 100 mL of 1 g·L⁻¹ ethephon solution or co-treatment of $C_2H_4+H_2S$ released from 150 mL of 1 mmol·L⁻¹ sodium hydrosulfide (NaHS) solution and 100 mL of 1 g·L⁻¹ solution ethephon at 23 ± 0.5 °C with relative humidity of 85% to 90%. After 1 d of storage, the solutions were replaced with distilled water. Each treatment unit comprised six tomatoes, and each experiment was repeated three times. The flesh of fruit without seeds was randomly sampled every other day and stored at -80 °C for subsequent experiments. The changes in tomato fruit color were measured with a color difference meter (model WSC-100; Konica Minolta, Tokyo, Japan), which provided CIE L*, a*, and b* values, where L* indicates lightness, a* indicates chromaticity on a green (-) to red (+) axis, and b* indicates chromaticity on a blue (-) to yellow (+) axis. Each fruit was measured at four equidistant points around the middle area.

Measurement of fruit firmness. The tomato fruit firmness was measured using a 2-mm-diameter flat probe with a texture analyzer (Model TA XT plus; Stable Micro Systems, Surrey, UK). Fruit firmness value was an average of 10 replicates with standard deviation.

Activity assay of cellulase, polygalacturonase, and pectin lyase. Frozen tissues (1 g) of tomato were homogenized with 4 mL of 8.8% NaCl containing 10 g·L⁻¹ polyvinyl pyrrolidone (PVPP). The mixture was centrifuged at 10,000 g_n for 20 min, and the supernatant was collected for enzyme activity assay.

Cellulase activity was determined as described by Abeles and Biles (1991). One unit

(U) of activity was defined as 1 µg of reducing sugar generated per 1 g of fresh weight (FW) per hour. The cellulase activity was expressed as U·g⁻¹ FW.

Polygalacturonase activity was determined according to Pathak and Sanwal (1998). One U of activity was defined as 1 µg of galacturonic acid generated per 1 g of FW per hour. The activity of polygalacturonase was expressed as U·g⁻¹ FW.

Pectin lyase was determined following the method described by Pitt (1988). The reaction was performed in a mixture of 1 mL of 1% pectin solution and 0.5 mL of enzyme extraction at 40 °C for 5 min and ended by adding 3.5 mL of 0.01 mol·L⁻¹ HCl. The absorbance was measured at 235 nm, and 1 U of activity was defined as an absorbance increase of 1.0 per min. The activity of pectin lyase was expressed as U·g⁻¹ FW.

Quantitative reverse-transcription polymerase chain reaction analysis. Candidate genes encoding cell wall-degrading enzymes, ethylene synthesis, and signaling genes were identified using a hidden Markov model search of protein domains obtained from the Pfam database (<http://pfam.xfam.org/>) and a local BLASTN analysis of genes (<https://solgenomics.net/>).

Total RNA from 0.1 g of frozen tomato fruit samples was extracted using the

RNA Extraction Kit (Tiangen, Beijing, China). Then, cDNA was synthesized using a reverse-transcription kit (PrimeScript RT Master Mix; Takara, Kyoto, Japan). The cDNA products were used for the quantitative polymerase chain reaction (qPCR) performed using Bio-Rad IQ5 (Hercules, CA) in a 10-µL reaction containing 5 µL of 2 × SYBR Premix Ex Taq (Takara, Kyoto, Japan), 0.8 µL of cDNA, 0.4 µL each for the forward and reverse primers, and 3.4 µL of ddH₂O. The specific primers used for the qPCR were designed based on the coding sequence of the genes as shown in the SGN database (<https://solgenomics.net/>) (Table 1). The qPCR was initiated using a denaturation step at 95 °C for 60 s, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 10 s. Expression of the actin gene in control tomatoes was used for normalization of data. The relative expression levels of all genes were calculated and analyzed by the 2^{-ΔΔCt} method. All analyses were repeated three times using three technical replicates with error bars.

Statistical analysis. Data were based on three replicates in each experiment, and the experiments were repeated independently three times. Statistical significance was tested using a one-way analysis of variance with IBM SPSS Statistics (SPSS version 20.0;

Table 1. Primers used for the quantitative polymerase chain reaction.

Primer	Sequence	Gene ID
<i>SILAT56</i> (forward)	5'-GCACTCGGGACAGCAATAAA-3'	Solyc03g058910
<i>SILAT56</i> (reverse)	5'-GTCACATCTCCAGCACTTGTG-3'	
<i>SILAT59</i> (forward)	5'-ATTAGGTTGACACAGGAGATG-3'	Solyc03g058890
<i>SILAT59</i> (reverse)	5'-GAGGGTTATACCAGCACCTTTAG-3'	
<i>SIPMEU1</i> (forward)	5'-CTCAAGGAAGAAGTACCCTAAC-3'	Solyc03g123630
<i>SIPMEU1</i> (reverse)	5'-TTCTGCACCGGTCTCAAATC-3'	
<i>SIPG2</i> (forward)	5'-GTGGTCCAGGTCATGGTATAAG-3'	Solyc10g080210
<i>SIPG2</i> (reverse)	5'-CCTGCCAAGTCTTGATCCTAA-3'	
<i>SIACS2</i> (forward)	5'-CCACTGGAGCTAATGAGACAAT-3'	Solyc01g095080
<i>SIACS2</i> (reverse)	5'-AATGCTGGGTAGTATGGTGAAG-3'	
<i>SIACS3</i> (forward)	5'-GACTTAGCCATGCGCAGATAA-3'	Solyc02g091990
<i>SIACS3</i> (reverse)	5'-CCCCTTGGAGAATGACTTCTT-3'	
<i>SIACS6</i> (forward)	5'-TGGAGAAAGTGAGAGGAGATAGA-3'	Solyc08g008130
<i>SIACS6</i> (reverse)	5'-ACCAGGATCAGCCAAACAA-3'	
<i>SIACO1</i> (forward)	5'-AAGGAAGTAGTGCAAGTAAGG-3'	Solyc07g049530
<i>SIACO1</i> (reverse)	5'-TAGGAAGATGGCGCAAGAG-3'	
<i>SIACO3</i> (forward)	5'-GATGGAAAGCCCTAGAGTTGAG-3'	Solyc09g089580
<i>SIACO3</i> (reverse)	5'-CCTTTAACACCGGCCTTAGT-3'	
<i>SIACO4</i> (forward)	5'-GCGCCATCTTCTACTTCTAAT-3'	Solyc02g081190
<i>SIACO4</i> (reverse)	5'-CTCAGCCAACCTTCTCCAATCT-3'	
<i>SIETR1</i> (forward)	5'-TGTGGAGCAACCATCTTATC-3'	Solyc12g011330
<i>SIETR1</i> (reverse)	5'-TCAAGGCCTTGCAGTAGTC-3'	
<i>SIETR2</i> (forward)	5'-CCGGAAGTTGCTATTGGAGAT-3'	Solyc07g056580
<i>SIETR2</i> (reverse)	5'-ACTGACACACTGCCTTCTTC-3'	
<i>SIETR3</i> (forward)	5'-CCTTTGGCAAGGATGAGGAATA-3'	Solyc09g075440
<i>SIETR3</i> (reverse)	5'-GACAGTTCAGCCAGTCATTAG-3'	
<i>SIETR4</i> (forward)	5'-GCCACGGCTATAACCCCTATTA-3'	Solyc06g053710
<i>SIETR4</i> (reverse)	5'-CTTCTCTACCAAGATCCCAAGTC-3'	
<i>SIETR5</i> (forward)	5'-ATGAGAGAGTCTGGAGCTATGA-3'	Solyc11g006180
<i>SIETR5</i> (reverse)	5'-AGAACAGTGGCATGGGATAAA-3'	
<i>SIETR6</i> (forward)	5'-GTGGTGTGGAGGTTCTGTTAG-3'	Solyc09g089610
<i>SIETR6</i> (reverse)	5'-AGACCCGAGGCATTCATCA-3'	
<i>SIERF1</i> (forward)	5'-CGCGTAATGGAATTAGGGTTTG-3'	Solyc05g051200
<i>SIERF1</i> (reverse)	5'-CCTCATTTGATAATGCGGCTTG-3'	
<i>SIERFD2</i> (forward)	5'-GGAGATGGTGACGGAGTTTAC-3'	Solyc12g056590
<i>SIERFD2</i> (reverse)	5'-CCGAGCTGGACGAAGAATAAG-3'	
<i>SIERF2</i> (forward)	5'-GTGGTGTGGAGATGAAGAAGAG-3'	Solyc09g075420
<i>SIERF2</i> (reverse)	5'-CGAATTTTCAGCAGCCCATTTAC-3'	
<i>SITubulin</i> (forward)	5'-TAGAGCCTGGTACGATGGATAG-3'	Solyc08g006890
<i>SITubulin</i> (reverse)	5'-CAACTCAGCGCCTTCAGTATAA-3'	

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Armonk, NY), and the results were expressed as the means $\pm$ sd. Significant differences were calculated following a significance ($P < 0.01$ or $P < 0.05$) t test. The Pearson correlation coefficient (R) was used to show the correlation among fruit firmness, cellulase activities, polygalacturonase (PG), pectin lyase (PL), expressions of softening-related genes, ethylene biosynthesis genes, ethylene receptor genes, and ethylene response factors of tomato fruit treated with C_2H_4 and $C_2H_4+H_2S$. The heatmap of the parameters was drawn by R script.

Results

Effects of H_2S and ethylene on the ripening and senescence of tomatoes during storage. To evaluate the potential impact of exogenous H_2S on fruit ripening, tomatoes at the mature green stage were treated with ethylene or ethylene+ H_2S for 1 d and subjected to normal conditions. As shown in Fig. 1A, fruits treated with ethylene showed obvious color breaking at 1 d and were fully red at 5 d. However, the ripening process was delayed by $C_2H_4+H_2S$ co-treatment, and tomatoes at the color breaking stage were still observed on day 5. Changes in the skin color of tomato fruits during storage were evaluated via L^* , a^* , and b^* . Figure 1B shows a loss of lightness in both treatment fruits, whereas the L^* value of fruits treated with $C_2H_4+H_2S$ decreased more slowly and was maintained at a higher value than that of fruits treated with C_2H_4 . Furthermore, the a^* values of fruits treated with $C_2H_4+H_2S$ increased slowly and were significantly lower than those of fruits treated with C_2H_4 during storage (Fig. 1C), suggesting that the color change from green to red was delayed in tomatoes treated with $C_2H_4+H_2S$.

Effects of H_2S and ethylene on the fruit firmness of tomatoes during storage. Fruit firmness is an important factor used to evaluate the quality of tomatoes. As shown in Fig. 2A, the firmness of the two groups decreased gradually from day 0 to day 5 during storage. However, ethylene+ H_2S resulted in significantly higher levels of fruit firmness compared with ethylene treatment during the whole storage period, suggesting that the co-treatment significantly delayed the ethylene-induced softening of tomato fruit.

Effects of H_2S and ethylene on the activities of cell wall-degrading enzymes in tomatoes during storage. To explore the role of H_2S in delaying tomato fruit softening, the activities of cell wall-degrading enzymes were determined in fruit. Figure 2B showed the changes of cellulase activities in tomatoes treated with ethylene and ethylene+ H_2S . Cellulase activity decreased dramatically on day 1, followed by a significant increase. Furthermore, ethylene+ H_2S treatment induced significantly low levels of cellulase activity on day 3 and day 5 compared with ethylene treatment. As shown in Fig. 2C, the activities of PG decreased gradually with both treatments. However, in comparison with the ethylene treatment, the ethylene+ H_2S

co-treatment apparently maintained lower PG activities from day 1 to day 5. Figure 2D shows that the activities of pectin lyase increased significantly on day 1, followed by a decrease. The activity of the ethylene+ H_2S co-treatment group of tomatoes on day 1 was 23.1% lower than that of ethylene treatment group of tomatoes. From day 3 to day 5, the pectin lyase activity of tomatoes administered co-treatment was

maintained at a significantly lower value ($P < 0.01$) than that of tomatoes treated with ethylene.

Effects of H_2S and ethylene on the expression of softening-related genes in tomatoes. Fruit softening is attributable to cell wall degradation caused by related enzymes; therefore, the expressions of softening-related genes are modulated during fruit ripening. As shown in Fig. 3A, the

Fig. 1. Color changes in tomato fruit treated with C_2H_4 and $C_2H_4+H_2S$ for 1 d (1 d of storage) and subsequently stored in normal conditions (3, 5, and 7 d of storage) (A). L^* indicates lightness (B), a^* indicates chromaticity on a green (-) to red (+) axis (C), and b^* indicates chromaticity on a blue (-) to yellow (+) axis (D).

Fig. 2. Effects of C_2H_4 and $C_2H_4+H_2S$ treatments on the firmness of tomato fruit (A) and the activities of cell wall-degrading enzymes cellulase (B), polygalacturonase (C), and pectin lyase (D). Fruit were fumigated with C_2H_4 and $C_2H_4+H_2S$ for 1 d and then stored in normal conditions in a 3-L chamber. Error bars indicate SE ($n = 10$ for fruit firmness and $n = 3$ for enzyme activities). Asterisks indicate significant differences between fruit treated with C_2H_4 and $C_2H_4+H_2S$ according to Student's t test (* $P < 0.05$; ** $P < 0.01$).

transcript level of the pectin lyase gene *SILAT56* increased gradually until day 5 with both treatments during the storage of tomatoes, and no significant difference was observed between ethylene and ethylene+H₂S treatment. Figure 3B shows the changes in another pectin lyase gene, *SILAT59*, in tomatoes. The expression of *SILAT59* increased slightly on day 1 in tomatoes with both treatments. On day 5 of storage, the transcript of *SILAT59* increased more than two-times that on day 1 with ethylene treatment. Compared with the ethylene group, the expression of *SILAT59* on day 5 with ethylene+H₂S cotreatment was significantly lower ($P < 0.01$) than that with ethylene treatment.

Figure 3C shows the changes in the expression of the pectin methyltransferase gene *SIPMEU1* in tomatoes during storage. The expression of *SIPMEU1* decreased gradually with both treatments, but no significant difference was observed between them. As shown in Fig. 3D, the expression of the polygalacturonase gene *SIPG2* increased dramatically during tomato storage, and it especially increased on day 5. The gene expression of *SIPG2* with ethylene treatment was significantly higher than that with the cotreatment on day 1 and day 5; however, an opposite trend was observed on day 3.

Effects of H₂S and ethylene on the expression of ethylene synthesis genes in tomatoes. Ethylene is the predominant phytohormone that promotes fruit ripening; therefore, the expressions of the genes involved in the biosynthesis of ethylene were analyzed. Figure 4 shows the expression patterns of the six ethylene biosynthesis genes, *SIACS2*, *SIACS3*, *SIACS6*, *SIACO1*, *SIACO3*, and *SIACO4*, in tomatoes treated with ethylene and with ethylene+H₂S. As shown in Fig. 4A, the expression of *SIACS2* increased and peaked on day 3, followed by a decrease with both treatments. However, ethylene+H₂S reduced *SIACS2* expression significantly on day 3 compared with ethylene treatment. Figure 4B shows that the expression of *SIACS3* fluctuated during tomato storage, and a significantly lower level of *SIACS3* expression was observed with cotreatment on day 3 in comparison with ethylene treatment alone. Figure 4C shows that the expression of *SIACS6* increased slightly with both treatments and that H₂S did not significantly affect the expression. Figure 4D shows that 1 d of fumigation with ethylene induced an increase of *SIACO1*, followed by a plateau. However, ethylene+H₂S treatment sustained significantly lower levels of *SIACO1* expression on day 1, followed by a peak on day 3. *SIACO1* expression with co-treatment was significantly lower on day 1 and day 5, but it was higher on day 3 compared with ethylene treatment alone. Figure 4E shows that ethylene treatment exhibited a continuous increase in *SIACO3* expression, whereas its expression with co-treatment increased slightly and peaked on day 3, followed by a decrease. Ethylene+H₂S induced a significantly lower level of *SIACO3* expression on day 1 and day

5 during tomato storage compared with ethylene treatment. Changes in patterns similar to those of *SIACO1* were observed for *SIACO4* expression during tomato storage. Ethylene induced an increase in *SIACO4* expression on day 1, but the increase was greatly attenuated with ethylene+H₂S cotreatment. The expression of *SIACO4* with cotreatment was significantly higher on day 3 and lower on day 5 compared with that with ethylene treatment. H₂S repressed the expression of ethylene synthesis genes *SIACS2*, *SIACS3*, *SIACO1*, *SIACO3*, and *SIACO4* in tomatoes during storage to different extents.

Effects of H₂S and ethylene on the expression of ethylene signaling genes in tomatoes. To evaluate the role of H₂S in ethylene signaling, the ethylene signaling genes including ethylene receptors and ethylene response factors were assayed at expression levels. As shown in Fig. 5A, the gene expression of *SIETR1* increased slightly with both ethylene and ethylene+H₂S treatment in tomatoes during storage, whereas the co-treatment induced a significantly lower expression of *SIETR1* on day 5 compared with ethylene alone. Similarly, the expression of *SIETR2* increased gradually with both treatments during storage, and H₂S was

Fig. 3. Relative gene expression patterns of softening-related genes pectin lyases *SILAT56* (A) and *SILAT59* (B) and pectin methyltransferase (*SIPMEU1*) (C) and polygalacturonase (*SIPG2*) (D) in tomato fruit treated with C₂H₄ and C₂H₄+H₂S for 1 d. Error bars indicate SE (n = 3). Asterisks indicate significant differences between fruit treated with C₂H₄ and C₂H₄+H₂S according to Student's *t* test (* $P < 0.05$; ** $P < 0.01$).

Fig. 4. Changes in the gene expression of ethylene biosynthesis-related genes of 1-aminocyclopropane-1-carboxylate synthase [*SIACS2* (A), *SIACS3* (B), and *SIACS6* (C)] and 1-aminocyclopropane-1-carboxylate oxidase [*SIACO1* (D), *SIACO3* (E), and *SIACO4* (F)] in tomato fruit during storage after C₂H₄ and C₂H₄+H₂S treatment for 1 d. Error bars indicate SE (n = 3). Asterisks indicate significant differences between C₂H₄ and C₂H₄+H₂S co-treated fruit according to Student's *t* test (* $P < 0.05$; ** $P < 0.01$).

found to attenuate the expression on day 5. Figure 5C shows that *SIETR3* increased with ethylene treatment on day 1, followed by a fluctuation. In addition, a significantly lower level of *SIETR3* expression with co-treatment compared with ethylene alone was observed on day 1 and day 5, but the trend was reversed on day 3. Figure 5D shows that *SIETR4* expression increased gradually with both treatments; however, a decrease was observed on day 5 with co-treatment. Furthermore, ethylene+H₂S treatment induced a higher expression of *SIETR4* on day 3 compared with ethylene treatment. Figure 5E depicts a trend of increasing expression that was observed with both ethylene and ethylene+H₂S treatment; however, ethylene+H₂S maintained a significantly lower expression level during the entire storage time compared with control. Figure 5F shows that ethylene treatment induced an increase of *SIETR6* on day 1, followed by a plateau, whereas ethylene+H₂S treatment attenuated the expression on day 1 and day 5. Ethylene+H₂S treatment was found to differentially affect the expression of ethylene receptor genes, and the expression levels of *SIETR1*, *SIETR2*, *SIETR3*, *SIETR5*, and *SIETR6* were significantly downregulated at day 5 in the cotreatment group.

Many ethylene response factors become involved in fruit ripening after sensing the signal of ethylene. As shown in Fig. 6A, ethylene treatment induced an increased expression of the ethylene responsive transcription factor *SICRF2* on day 1, followed by a plateau, whereas ethylene+H₂S treatment significantly inhibited the expression on day 1 and day 3. Figure 6B shows that *SIERF1* increased slightly with both treatments; however, no significant difference was observed. Generally, the trend of increased expression of *SIERF2D* was observed with both treatments, but H₂S caused no significant changes in the expression (Fig. 6C). Figure 6D shows that *SIERF2* changed little during storage with both treatments, but ethylene+H₂S treatment maintained a significantly lower level of expression during the whole storage time.

Correlation analysis and heatmap of the values determined in tomatoes during storage. To determine the factors contributing to tomato fruit softening, the correlation among the value of firmness, activities of cellulase, polygalacturonase, pectin lyase, expressions of softening-related genes (*SILAT56*, *SILAT59*, *SIPMEU1*, and *SIPG2*), ethylene biosynthesis genes (*SIACS2*, *SIACS3*, *SIACS6*, *SIACO1*, *SIACO3*, and *SIACO4*), ethylene receptor genes (*SIETR1*, *SIETR2*, *SIETR3*, *SIETR4*, *SIETR5*, and *SIETR6*), and ethylene response factors (*SICRF2*, *SIERF1*, *SIERFD2*, *SIERF2*) were analyzed. As shown in Fig. 7, the data indicated that fruit firmness was negatively correlated with the expression of ethylene-related genes *SIACOs*, *SIACs*s, *SIETR*s, and *SIERF*s in tomato fruits. Similarly, tomato fruit firmness was negatively correlated with the expression of softening-related genes *SILAT56*, *SILAT59*, and *SIPG2*.

Fig. 5. Changes in the gene expression of ethylene receptor-related genes *SIETR1* (A), *SIETR2* (B), *SIETR3* (C), *SIETR4* (D), *SIETR5* (E), and *SIETR6* (F) in tomato fruit during storage after C₂H₄ and C₂H₄+H₂S treatment for 1 d. Error bars indicate SE (n = 3). Asterisks indicate significant differences between fruit treated with C₂H₄ and C₂H₄+H₂S according to Student's *t* test (* *P* < 0.05; ** *P* < 0.01).

Fig. 6. Changes in the gene expression of ethylene response factors *SICRF2* (A), *SIERF1* (B), *SIERFD2* (C), and *SIERF2* (D) in tomato fruit during storage after C₂H₄ and C₂H₄+H₂S treatment for 1 d. Error bars indicate SE (n = 3). Asterisks indicate significant differences between C₂H₄ and C₂H₄+H₂S cotreated fruit according to Student's *t* test (* *P* < 0.05; ** *P* < 0.01).

The heatmap of the physiological parameters of tomatoes during storage was drawn (Fig. 8) to show the difference between ethylene and ethylene+H₂S treatment. It could be observed that ethylene+H₂S alleviated the softening of tomatoes by attenuating the activities of cell wall-degrading enzymes and their expression, and also the expression of ethylene biosynthesis and signaling genes.

Discussion

Fruit ripening is a complex developmental process that is coordinated with the activities of softening-related enzymes and the upregulation of a large set of ripening-related genes. It has

been reported that fruit softening is a significant characteristic of fruit ripeness and an important factor affecting tomato fruit quality, storage, and shelf life (Oms-Oliu et al., 2011). Our results showed that the treatment of tomato fruit with exogenous H₂S significantly delayed the fruit softening process and inhibited the activity of softening enzymes. The transition of tomato color from green to red was delayed by H₂S compared with ethylene alone. Tomatoes were fumigated with H₂S released from 1 mM of NaHS or NaHS+ethylene for 1 d and subjected to normal conditions. According to our previous studies, the NaHS concentration used in the present research was within the safety range.

Fig. 7. Correlation analysis among the value of firmness, activities of cellulase, polygalacturonase (PG), pectin lyase (PL), expressions of softening-related genes (*SILAT56*, *SILAT59*, *SIPMEU1*, and *SIPG2*), ethylene biosynthesis genes (*SIACS2*, *SIACS3*, *SIACS6*, *SIACO1*, *SIACO3*, and *SIACO4*), ethylene receptor genes (*SIETR1*, *SIETR2*, *SIETR3*, *SIETR4*, *SIETR5*, and *SIETR6*), and ethylene response factors (*SICRF2*, *SIERF1*, *SIERFD2*, *SIERF2*) in tomato fruit treated with C_2H_4 and $C_2H_4+H_2S$. Pearson's correlation coefficient among data were analyzed using R scripts.

Fig. 8. Heatmap based on the value of firmness, activities of cellulase, polygalacturonase (PG), pectin lyase (PL), expressions of softening-related genes (*SILAT56*, *SILAT59*, *SIPMEU1*, and *SIPG2*), ethylene biosynthesis genes (*SIACS2*, *SIACS3*, *SIACS6*, *SIACO1*, *SIACO3*, and *SIACO4*), ethylene receptor genes (*SIETR1*, *SIETR2*, *SIETR3*, *SIETR4*, *SIETR5*, and *SIETR6*), and ethylene response factors (*SICRF2*, *SIERF1*, *SIERFD2*, *SIERF2*) in tomato fruit treated with C_2H_4 and $C_2H_4+H_2S$. Co. indicates treatment with $C_2H_4+H_2S$. Heatmap scale bars are shown in the right part of the figure. Pearson's correlation coefficient among data were analyzed using R scripts.

Many enzymes are associated with softening in fruits, including pectinmethylesterase, polygalacturonase, cellulase, and others (Brummell and Harpster, 2001). Polygalacturonase and cellulase are required for the modification of pectin and cellulase, respectively. To alleviate fruit softening, various postharvest measures have been applied, such as ultraviolet-C illumination (Bu et al.,

2013) and melatonin treatment (Zhai et al., 2018). In the present study, we found that exogenous H_2S alleviated ethylene-induced fruit softening. Furthermore, the enzymes required for cell wall-degrading cellulase, polygalacturonase, and pectin lyase were found to be attenuated by H_2S fumigation. Gene expression data consistently showed that the genes encoding pectin lyases

(*SILAT56* and *SILAT59*), pectin methylesterase (*SIPMEU1*), and polygalacturonase (*SIPG2*) were downregulated in tomato fruit treated with H_2S for 1 d. Among the enzymes, PG showed very high activity during tomato fruit softening, suggesting that PG might be the key enzyme for cell wall degradation (Brummell and Labavitch, 1997). It was found that the gene *SIPG2* was significantly upregulated at a late stage of storage, whereas H_2S inhibited the increase (Fig. 3D). The results implied that exogenous H_2S alleviated tomato fruit ripening by attenuating the activities of cell wall degradation enzymes and their gene expressions.

Tomato is typically a climacteric fruit, and its ripening and senescence are accompanied by the synthesis of large amounts of ethylene. Therefore, the control of ethylene biosynthesis or its signaling pathway can be applied to alleviate tomato fruit ripening and senescence. For instance, 1-MCP, an ethylene receptor inhibitor, is broadly used during postharvest fruit storage (Song et al., 2018; Watkins et al., 2000), and acetylsalicylic acid shows inhibitory effects on ethylene biosynthesis and signaling during kiwifruit ripening (Yin et al., 2013). In the present research, we studied the regulatory role of exogenous H_2S on the expression of ethylene synthesis and signal transduction-related genes in tomatoes. ACC synthase and ACC oxidase are the enzymes that catalyze the conversion of AdoMet to ACC and ACC to ethylene, respectively. It was found that H_2S significantly reduced the expression of *SIACS2* and *SIACS3*, whereas *SIACS4* genes remained unchanged compared with ethylene. Ethylene significantly upregulates the expression of *SIACO1*, *SIACO3*, and *SIACO4* after 1 d of treatment, whereas H_2S attenuated the increase, suggesting that H_2S might inhibit ethylene synthesis at the expression level. These results were consistent with those of our previous report of the role of H_2S in alleviating the ripening and senescence of kiwifruit and banana (Ge et al., 2017; Li et al., 2017). The expression patterns of ethylene receptor genes showed that H_2S treatment differentially affected their expressions, and expression levels of *SIETR1*, *SIETR2*, *SIETR3*, *SIETR5*, and *SIETR6* were significantly downregulated by H_2S . After being recognized by ethylene receptors and a signaling cascade, ethylene has physiological roles in a large family of ERFs, subsequently inducing changes in the metabolism involved in fruit ripening and senescence (Bapat et al., 2010). The present results showed that *SICRF2* and *SIERF2* were downregulated by H_2S treatment. Exogenous H_2S was found to antagonize the synthesis and signaling of ethylene, thereby alleviating fruit ripening in tomatoes.

Texture softening is a predominant characteristic of ethylene-induced fruit ripening. We found that H_2S could attenuate the expression of ethylene signaling and pathway genes. The cell wall degradation enzymes (CWDE) was also decreased by H_2S treatment. Therefore, the correlation was analyzed and the results showed that fruit

firmness was negative correlated with the expression of ethylene-related genes *SIACO*s, *SIACS*s, *SIETRs*, and *SIERFs* in tomato fruits. It is difficult to say whether H₂S regulates these two events separately or in a linear way. Accumulating reports found that ethylene can act upstream of CWDE. For instance, the expression of CWDE is ethylene-dependent; therefore, fruit softening and cell wall disassembly are regulated by ethylene in Charentais melon (Nishiyama et al., 2007). The ERF DkERFs in persimmon fruit was found to directly bind to the promoter of a CWDE gene *DkXTH9* and regulate its gene expression (Wang et al., 2017). Therefore, the role of H₂S in regulating fruit softening could be as a linear signaling pathway (H₂S-ethylene-CWDE).

Short-term application of H₂S alleviates fruit softening by inhibiting the activities and gene expressions of cell wall-degrading enzymes. This study also demonstrated that H₂S downregulates ethylene biosynthesis and signaling genes, effectively protecting cell wall components from being degraded. This work provides insight into the multifaceted role of H₂S in fruit senescence and implicates the potential application of H₂S during postharvest fruit storage.

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