

A histone deacetylase gene, *SIHDA3*, acts as a negative regulator of fruit ripening and carotenoid accumulation

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Abstract

Key message *SIHDA3* functions as an inhibitor and regulates tomato fruit ripening and carotenoid accumulation.

Abstract Post-translational modifications, including histones acetylation, play a pivotal role in the changes of chromatin structure dynamic modulation and gene activity. The regulation of histone acetylation is achieved by the action of histone acetyltransferases and deacetylases, which play crucial roles in the regulation of transcription activation. There is an increasing research focus on histone deacetylation in crops, but the role of histone deacetylase genes (*HDACs*) in tomato has not been elucidated. With the aim of characterizing the tomato RPD3/*HDA1* family histone deacetylase genes, *SIHDA3* was isolated and its

RNA interference (RNAi) lines was obtained. The fruit of *SIHDA3* RNAi lines exhibited accelerated ripening process along with short shelf life characteristics. The accumulation of carotenoid was increased due to the alteration of the carotenoid pathway flux. Climacteric ethylene production also stimulated along with significantly up-regulated expression of ethylene biosynthetic genes (*ACS2*, *ACS4*, *ACO1* and *ACO3*) and fruit ripening-associated genes (*RIN*, *E4*, *E8*, *PG*, *Pti4*, *LOXB*, *Cnr* and *TAGL1*) in *SIHDA3* RNAi lines. Besides, fruit cell wall metabolism-associated genes (*HEX*, *MAN*, *TBG4*, *XTH5* and *XYL*) were enhanced in transgenic lines. Relative to wild type (WT) plants, *SIHDA3* RNAi seedlings displayed shorter hypocotyls and more sensitivity to ACC (1-aminocyclopropane-1-carboxylate). These results indicated that *SIHDA3* is involved in the regulation of fruit ripening by affecting ethylene biosynthesis and carotenoid accumulation.

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Keywords Carotenoid · Ethylene · Fruit ripening · Histone deacetylases · RNAi · Tomato

Abbreviations

<i>HDACs</i>	Histone deacetylase genes
ACC	1-Aminocyclopropane-1-carboxylate
ACS	1-Aminocyclopropane-1-carboxylate synthase
ACO	1-Aminocyclopropane-1-carboxylate oxidase
B	Breaker
<i>CYC-B</i>	Chromoplast-specific lycopene- β -cyclase
DPA	Days post-anthesis
<i>LCY</i>	Chloroplast-specific lycopene- β -cyclase
IMG	Immature green
MG	Mature green
ORF	Open reading frame
<i>PSY1</i>	Phytone synthase 1
RACE	Rapid amplification of cDNA ends
RNAi	RNA interference
WT	Wild type

Introduction

The regulation of the positive/negative transcription and modulation of chromatin structure are mediated by the reversible process of histone acetylation and deacetylation (Berger 2002). Histone acetylation and deacetylation are usually associated with the transcription activation and repression, respectively. Their regulation is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs).

The HDACs in plants are divided into three subfamilies based on their sequence similarity and the dependency on co-factor: RPD3/HDA1, which is most closely related to the yeast and common in eukaryotes (Hollender and Liu 2008); HD2, which is plant specific and originated in maize (Lusser et al. 1997; Pandey et al. 2002; Yang and Seto 2007); and SIR2, a nicotinamide adenine dinucleotide (NAD)-dependent enzyme (Frye 2000). Fourteen HDACs have been identified in tomato: nine are RPD3/HDA1 subfamilies (SIHDA1-9); three are HDT subfamilies (SIHDT1, SIHDT2 and SIHTD3) and two are SIR2 subfamilies (SIR1 and SIR2). The RPD3/HDA1 subfamilies are subdivided into four classes: class I (SIHDA1-SIHDA4), class II (SIHDA7-SIHDA9), class III (SIHDA5) and class IV (SIHDA6) (Cigliano et al. 2013b; Guo et al. 2017). Studies performed in different plant species have shown that histone acetylation is associated with several aspects of development, especially in *Arabidopsis*. For instance, *AtHDA6* found to be involved in maintaining DNA methylation (Aufsatz et al. 2002; Probst et al. 2004) and light signaling pathway (Tessadori et al. 2009); *AtHDA6* and *AtHDA19* act as

suppressors in embryonic properties after germination (Tanaka et al. 2008); *AtHDA7* is required for the seed germination, plant growth, female gametophyte development and embryogenesis (Cigliano et al. 2013a); however, relatively few *HDACs* that have been characterized in tomato. Several studies suggested a connection between tomato fruit ripening and histone deacetylation (Cigliano et al. 2013b; Zhao et al. 2014). Nevertheless, they have not been experimentally studied and reported in tomato to date.

Fruit ripening is a complex regulated process that involves numerous metabolic changes, such as changes in color, flavor, aroma and nutrition. The process is controlled by endogenous hormonal as well as genetic regulators and external signals (temperature, light and hydration). Ripening allows fruit to facilitate seed dispersal and provides essential nutrition in the human diet (Klee and Giovannoni 2011). Tomato is categorized as a climacteric fruit based on the dramatic increase in respiration rate and ethylene production during ripening (Alexander and Grierson 2002). In the ripening processes of climacteric fruits, ethylene is an essential factor, as it was illustrated through the analysis of the fruit ripening progress in the ethylene-suppressed transgenic plants and the tomato ethylene receptor mutant *Nr* (*never ripe*) (Wilkinson et al. 1995; Moore et al. 2002).

Tomato is usually considered to be an excellent model plant for studying climacteric fruit ripening. To date, the regulatory mechanisms controlling fruit ripening in tomato have been studied extensively. In these studies, a series of natural ripening-deficient mutants, such as *rin*, *Nr*, *Cnr* and *TAGL1*, have facilitated our understanding of the transcriptional control system underlying tomato ripening (Vrebalov et al. 2002; Wilkinson et al. 1995; Manning et al. 2006).

During ethylene biosynthesis, the ethylene perception and response abilities are essential for fruit ripening. The expression of *E4* in fruit is up-regulated following the induction of ethylene (Xu et al. 1996). Meanwhile, *E4* in fruit is highly suppressed through ethylene biosynthesis inhibition (Lincoln and Fischer 1988). The expression of *E8*, a ripening-associated and fruit-specific gene, is also regulated by ethylene in tomato (Deikman and Fischer 1988). Illuminating the relationship between the mechanism of fruit ripening and the regulation of these gene activities is important for us to understand the processes of ripening.

Here, the functional characterization of an *HDAC* gene, *SIHDA3*, isolated from tomato fruits based on a cDNA clone is reported. A previous report indicated that *SIHDA3* is mainly expressed in fruit and its transcript increases along with fruit development and ripening (Cigliano et al. 2013b). However, to date, *SIHDA3* has not been studied for

its functional attributes in tomato. In this study, RNAi repression of *SIHDA3* was performed to investigate the exact role of *SIHDA3* in tomato. The results confirmed the function of *SIHDA3* as an inhibitor of fruit ripening.

Materials and methods

Plant materials and growth conditions

In this study, WT plants (*Solanum lycopersicum* Mill. cv. Ailsa Craig) and transgenic plants were planted in a greenhouse for 16 h days (25 °C), 8 h nights (18 °C) and watered daily. The flowers were labeled at anthesis and fruit development was recorded as days post-anthesis (DPA). According to days post-anthesis and the color of fruit, the tomato fruits ripening stages were subdivided into five stages: IMG (immature green; 20 DPA), MG (mature green; 33 DPA, full size fruits expansion and the development accomplished but no obvious color change), B (breaker; 36DPA, the fruits color changes from green to yellow), B + 4 (4 days after breaker) and B + 7 (7 days after breaker). All plant samples were collected, immediately frozen in liquid nitrogen and stored at −80 °C for further analysis.

Cloning of *SIHDA3*

Total RNA was isolated from all plant tissues including root, stem, leaf (young leaf, mature leaf and senescent leaf), flower, sepal and different fruit developmental stages of WT and homozygous T2 transgenic tomato plants using Trizol (Invitrogen, USA), according to the manufacturer's instructions. Then, 2 µg of total RNA was used to synthesize first-strand cDNA using the reverse transcription polymerase chain reaction (M-MLV reverse transcriptase, Takara) with an Oligo(dT)₁₈ primer. A 1–2 µL cDNA template was used to clone the full length *SIHDA3* gene with primers FHDA3-F and FHDA3-R (Supplementary Table S1) using high fidelity PCR (Prime STARTTM HS DNA polymerase, Takara). Positive clones were picked out via *Escherichia coli* JM109 transformation and confirmed by sequencing (Invitrogen).

Construction of the *SIHDA3* RNAi vector and plant transformation

To further study the function of the *SIHDA3* gene, an RNAi vector was constructed. The primers of SIHDA3-RNAi-F and SIHDA3-RNAi-R (Supplementary Table S1) were used to amplify the specific DNA fragment of *SIHDA3* gene. The amplified products of 409 bp were purified and digested with *HindIII/XbaI* and *XhoI/KpnI* restriction sites, respectively. Then, the digested products were linked into the

pHANNIBAL plasmid at the *HindIII/XbaI* restriction site in the sense orientation and at the *XhoI/KpnI* restriction site in the antisense orientation. Finally, the double-stranded RNA expression unit, which includes the cauliflower mosaic virus 35S promoter, the *SIHDA3* fragment in the antisense orientation, a PDK intron, the *SIHDA3* fragment in the sense orientation, and the OCS terminator, was digested with *SacI* and *SpeI* (the isocaudamer of *XbaI*) and inserted into the plant binary vector pBIN19 using *SacI* and *XbaI* restriction sites. The resulting construct was transformed into tomato cv. Ailsa Craig using *Agrobacterium tumefaciens* (strain LBA4404) by the freeze–thaw method (Zhu et al. 2014). Transformed lines were selected by kanamycin resistance (80 mg L^{−1}) resistance and analyzed by the primers NPTII-F/R (Supplementary Table S1). The positive transgenic plants were picked out and used for subsequent experiments.

Quantitative RT-PCR (qPCR) analysis

The synthesized cDNAs were diluted two times with RNase/DNase-free water. The qPCR analysis was performed using the CFX96TM Real-Time System (C1000TM Thermal Cycler, Bio-Rad). All reactions were carried out using the SYBR[®] Premix Go Taq II kit (Promega, China) in a 10 µL total sample volume (5.0 µL of 2× SYBR Premix Go Taq, 0.5 µL of primers, 1.0 µL of cDNA, 3.5 µL of ddH₂O). For analysis of each gene, an NRT (no reverse transcription control) and NTC (no template control) were also performed. The tomato *SICAC* and *SIEF1α* genes were used as internal standards for development studies and abiotic stress studies, respectively (Expósito-Rodríguez et al. 2008; Nicot et al. 2005). The relative gene expression levels were conducted using the 2^{−ΔΔC_T} method (Livak and Schmittgen 2001). Primers used for qPCR experiment are shown in Supplementary Table S2. Three biological replicates and three technical replicates were used for RT-PCR analyses, respectively.

Ethylene measurements

Fruits in different stages, including B, B + 4 and B + 7 stages, were harvested and placed in open 100 mL jars for 3 h to minimize the effects on ethylene production by the fruit picking. The jars were sealed and put on the room temperature for 24 h to collect the ethylene gas. Then, 1 mL of headspace gas was injected into a Hewlett-Packard 5890 series gas chromatograph equipped with a flame ionization detector. Samples were compared with standards of known concentration and normalized for fruit weight (Chung et al. 2010). Three biological replicates and three technical replicates were used for ethylene measurements.

Pigment quantification in tomato fruit

Pigments from tomato pericarp were extracted using a modified protocol from the previous report (Forth and Pyke 2006). A 1.0 g sample was cut from the pericarp of MG, B, B + 4 and B + 7 of WT and RNAi lines, respectively. Then, samples were grounded in the presence of liquid nitrogen and mixed with 20 mL of 60:40% (v/v) hexane:acetone. The extract was centrifuged at $4000\times g$ for 5 min and the supernatant was carefully transferred to a new tube. The sediment was repeatedly extracted with fresh solvent until colorless and the absorbance of supernatant was measured at 450, 647 and 663 nm, respectively. The total Chl and carotenoid contents were calculated with the following equations: total Chl $\text{mg mL}^{-1} = 8.02 (\text{OD}_{663}) + 20.2 (\text{OD}_{647})$ and total carotenoids $\text{mg mL}^{-1} = (\text{OD}_{450})/0.25$. Individual tissue samples were taken from 3 to 4 fruits for each ripening stage in biological triplicate and three technical replicates.

Postharvest storage test

The fruits of WT and RNAi lines were harvested at the B + 7 stage, and placed on filter paper in greenhouse conditions for 16 h days (25 °C) and 8 h nights (18 °C). The phenotype was observed every 2 days (Xie et al. 2014).

Ethylene triple response assay

The WT seeds were sterilized and sown on MS medium with 0, 1.0, 2.0, 5.0, 10.0, and 20.0 μM ACC. However, T2 seeds of RNAi lines were sterilized and sown on MS medium with 0, 5.0 and 10.0 μM ACC. After that, all seeds were cultured and cultured in the dark at 25 °C. Then, we measured the length of hypocotyl and root at least 20 seedlings after 7 days sowing. The expression of *ACO1*, *ACO3*, *ACS2* and *ACS4* in the seedlings of WT and transgenic lines was assessed to further explore the molecular mechanism of the triple response. The expression of *SIHDA3* was also detected in WT seedlings treated with 0, 1.0, 2.0, 5.0, 10.0, and 20.0 μM ACC.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and differences between means were defined as significant by a *t* test with $P < 0.05$.

Results

SIHDA3 impacts fruit ripening

To further study the function of tomato *SIHDA3* gene in fruit development and ripening, an RNAi vector of *SIHDA3*

was constructed to suppress its transcription in tomato plants. Seven independent transgenic lines of *SIHDA3* RNAi were obtained and selected for characterization. The expression level of *SIHDA3* in leaves was significantly declined in all transgenic events, especially those of RNAi2 and RNAi5, exhibiting approximately 90% reduction (Fig. 1a). To further verify the impact of *SIHDA3* repression, total RNA was isolated from fruit of MG, B, B + 4 and B + 7 stages of WT and transgenic tomato. As shown in Fig. 1b, the transcript level of *SIHDA3* was significantly reduced to roughly 3–5% of control levels during B to B + 7 stages in the RNAi lines, while only about 90% of silencing efficiency was reached in MG stage. Nevertheless, the expression levels of *SIHDA1* and *SIHDA2*, two homologs of *SIHDA3*, were not affected in *SIHDA3* RNAi lines (Fig. 1c, d), suggesting that the RNAi construct targeting *SIHDA3* is specific and does not target other HDAC genes.

Although *AtHDA6*, the closest homolog gene to *SIHDA3* in *Arabidopsis*, was shown to affect leaf senescence and late-flowering (Wu et al. 2008), no similar changes (data not shown) were observed in *SIHDA3* RNAi plants. However, obvious alterations in transgene lines were detected during fruit development and ripening. The time from anthesis to ripening stages was measured and relative to WT fruits, the ripening time occurred 2–3 days earlier in RNAi lines (Table 1). Similarly, color changes were observed earlier in *SIHDA3* RNAi fruits compared with those of WT (Fig. 1e).

Reduced expression of *SIHDA3* alters the fruit carotenoid and Chl accumulation profile

It was reported that the dramatic color change from green to red in tomato fruits is caused by the degradation of chlorophyll and the accumulation of carotenoids, including lycopene (red) and β -carotene (orange) (Giovannoni 2001; Fraser et al. 1994). In this study, total chlorophyll and carotenoids in the RNAi lines and WT fruits at B, B + 4 and B + 7 stages were measured. As shown in Fig. 2a, a 15–20% decrease of total Chl was observed in RNAi lines of fruits at the B stage, and decreased by approximately 50–60% in transgenic lines compared with WT fruits at the B + 4 and B + 7 stage. In contrast, the total carotenoids increased by 5–15% in RNAi fruits compared with WT fruits (Fig. 2b).

To confirm the underlying causes of the differences in color and carotenoid accumulation between the *SIHDA3* RNAi lines and WT, the expression levels of carotenoid biosynthesis-related genes were assessed in the fruit pericarp of *SIHDA3* RNAi lines and WT from MG to B + 7 stages (Fig. 3). The results displayed that *PSY1* (phytyl synthase 1) was up-regulated in RNAi fruits, while the

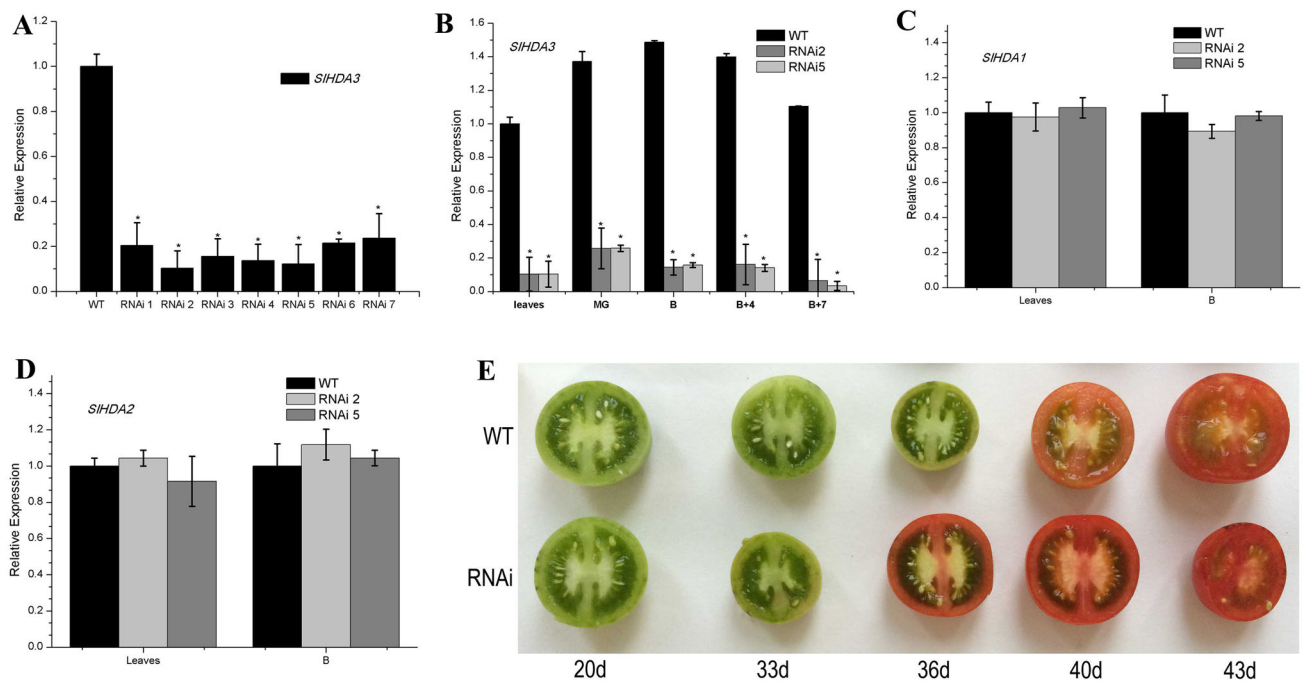


Fig. 1 Phenotypic and gene expression analyses of *SIHDA3* in RNAi lines. **a** Expression of *SIHDA3* in RNAi lines and WT. RNAs were extracted for qPCR assay from leaves of RNAi lines and the WT. Three replications for each sample were performed. **b** Relative expression profiles of *SIHDA3* between WT and *SIHDA3* RNAi lines. Data are the mean $\pm$ SD of three independent experiments. The WT

expression data in leaves are normalized to 1. **c, d** Other two *SIHDAC* genes expression in *SIHDA3* RNAi lines and WT fruits. **e** Fruits phenotype of WT and *SIHDA3* RNAi lines. 20–43 days, statistical time starting from the pollination. *SIHDA3* RNAi lines changed earlier 2–3 days than WT. The asterisks indicate statistically significant differences between the WT and transgenic fruits ($P < 0.05$)

Table 1 Days from anthesis to breaker stage for WT and *SIHDA3* RNAi lines

Tomato line	Days
WT	36.0 $\pm$ 0.50
RNAi 2	33.0 $\pm$ 0.50
RNAi 5	33.3 $\pm$ 0.42
RNAi 7	34.7 $\pm$ 0.44

expression of *CYC-B*, *LCY-B* and *LCY-E* was remarkably down-regulated in RNAi fruits compared with WT.

Reduced expression of *SIHDA3* affects ethylene synthesis and ethylene-related gene expression during ripening

Ethylene biosynthesis, perception and signal transduction are essential for the initiation and completion of tomato fruit ripening (Alexander and Grierson 2002), and the accumulation of carotenoid is also regulated by ethylene (Mauders et al. 1987). The assay of ethylene production measurement indicated that *SIHDA3* repression induced ethylene production in transgenic lines compared with the WT at the B + 4 and B + 7 stages (Fig. 4a).

Furthermore, the transcript levels of ethylene biosynthesis-related genes *ACO1*, *ACO3*, *ACS2* and *ACS4* and signal transduction gene *ERF1* were detected in pericarp

from the MG to B + 7 stages in the transgenic lines (line 2 and line 5) fruits and WT (Fig. 4b–f). All these ethylene biosynthetic genes exhibited remarkably up-regulated compared with WT fruits.

To measure the ethylene sensitivity of *SIHDA3* RNAi plants, the ethylene triple response assay was carried out. The seeds of WT and *SIHDA3* RNAi were sown on MS medium with or without ACC, the precursor of ethylene, which can be absorbed by the roots and translated into ethylene rapidly. The length of hypocotyls and roots was measured 7 days after sowing. The results showed that the average lengths of hypocotyls in RNAi lines have no difference with that of WT in the absence (0 μ M) of ACC, but there were significantly shorter in the presence of ACC (5.0 and 10.0 μ M) (Fig. 5a, c). In addition, the average lengths of roots in transgenic lines and WT seedlings were nearly identical in the absence (0 μ M) of ACC, but RNAi seedlings had longer roots than WT at higher levels of ACC (5.0 and 10.0 μ M) (Fig. 5a, b).

Subsequently, the expression of ethylene-related genes was also detected in transgenic lines and WT seedlings. The results showed that *ACO1*, *ACO3*, *ACS2* and *ACS4* were significant up-regulated in RNAi seedlings in the presence of ACC (5.0 μ M) (Fig. 5d). In addition, the transcript of *SIHDA3* in WT seedlings decreased dramatically after ACC treatment (Fig. 5e).

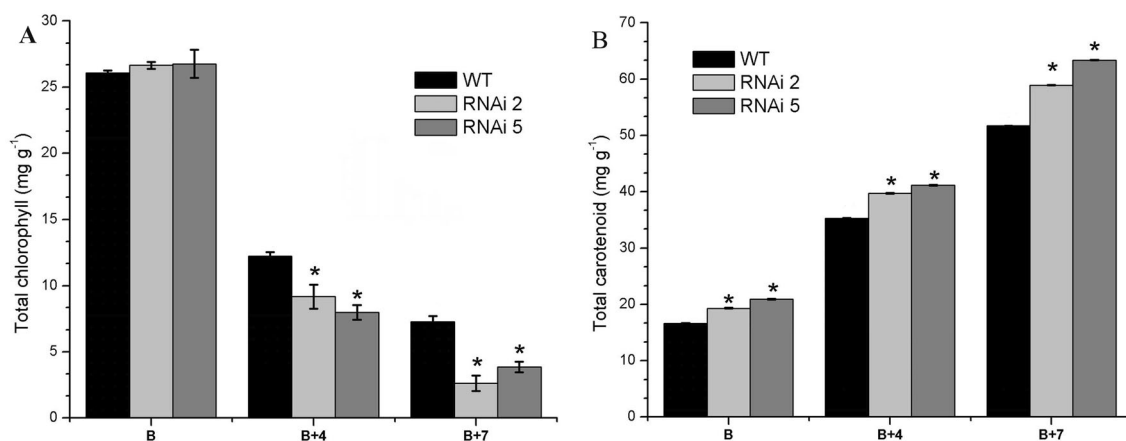


Fig. 2 Chl (a) and carotenoid (b) accumulation profiles between WT and *SIHDA3* RNAi fruits in pericarp. *B* breaker, *B* + 4 4 days after breaker stage, *B* + 7 7 days after breaker stage. Biological replicates (3–4 fruits per fruit ripening stage) were performed in triplicate, and

the data are presented as mean $\pm$ SD. The asterisks indicate statistically significant differences between the WT and transgenic fruits ($P < 0.05$)

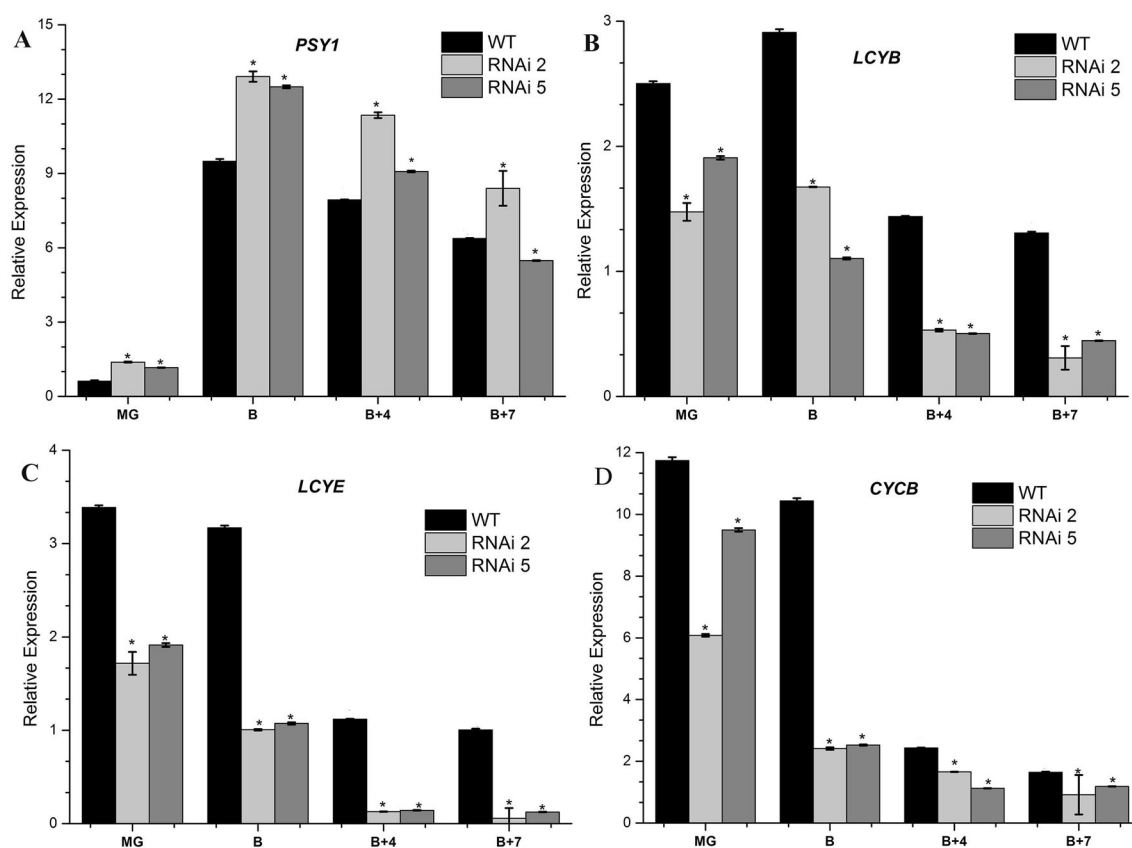


Fig. 3 Expression of carotenoid biosynthesis genes in pericarp between WT and *SIHDA3* RNAi lines. *MG* mature green, *B* breaker, *B* + 4 4 days after breaker stage, *B* + 7 7 days after

breaker stage. Data are the mean $\pm$ SD of three independent experiments. The asterisks indicate statistically significant differences between the WT and transgenic fruits ($P < 0.05$)

Expression analysis of ripening-associated genes in *SIHDA3* silenced fruits

Given that *SIHDA3* gene is highly expressed at the beginning of fruit ripening and the accelerated fruit ripening phenotype

of *SIHDA3* RNAi fruits, the expression of some known ripening-associated genes was examined in pericarp in WT fruit and *SIHDA3* RNAi lines (2 and 5). Figure 6a–c, g, h shows that the transcription of *RIN*, *E4*, *E8*, *Cnr* and *TAGL1* was markedly increased in the RNAi fruits.

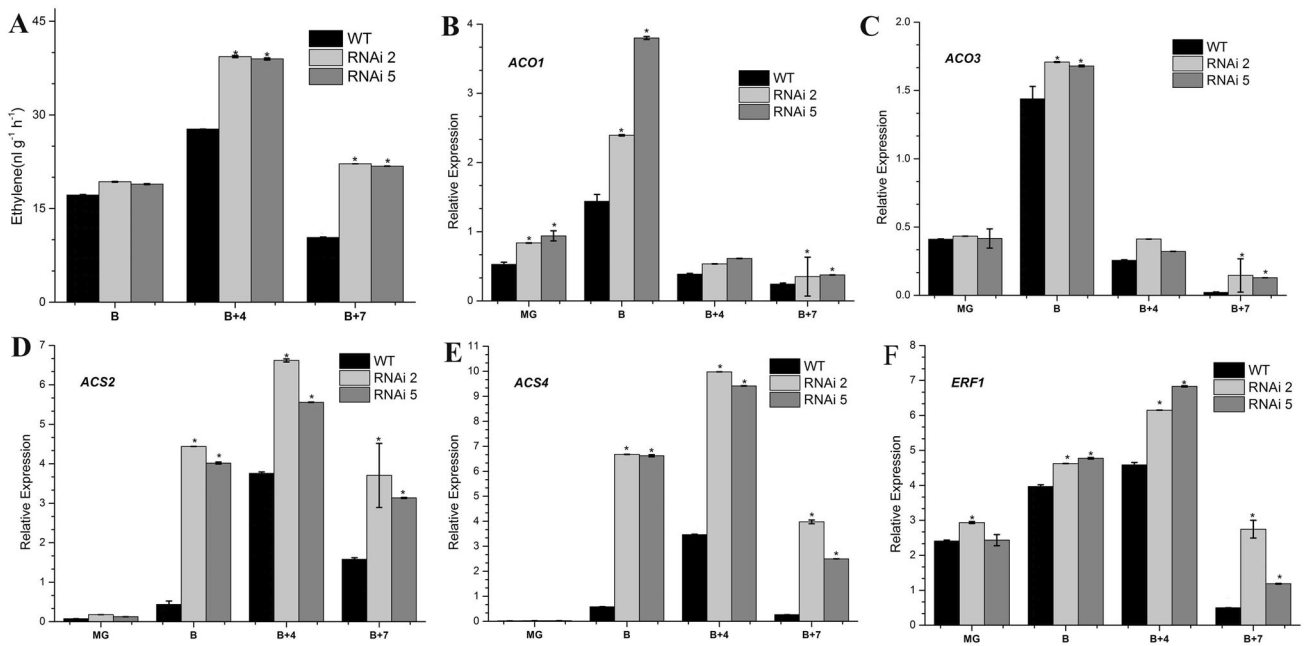


Fig. 4 **a** Ethylene production and **b–f** relative expression profiles of ethylene-related genes in the pericarp between WT and *SIHDA3* RNAi fruits. Ethylene production of WT and transgenic fruits was detected at the indicated stage (B, B + 4 and B + 7). Data are the mean $\pm$ SD of at least three individual fruits. MG mature green,

B breaker, B + 4 4 days after breaker stage, B + 7 7 days after breaker stage. Gene relative expression data are the mean $\pm$ SD of three independent experiments. The asterisks indicate statistically significant differences between the WT and transgenic fruits ($P < 0.05$)

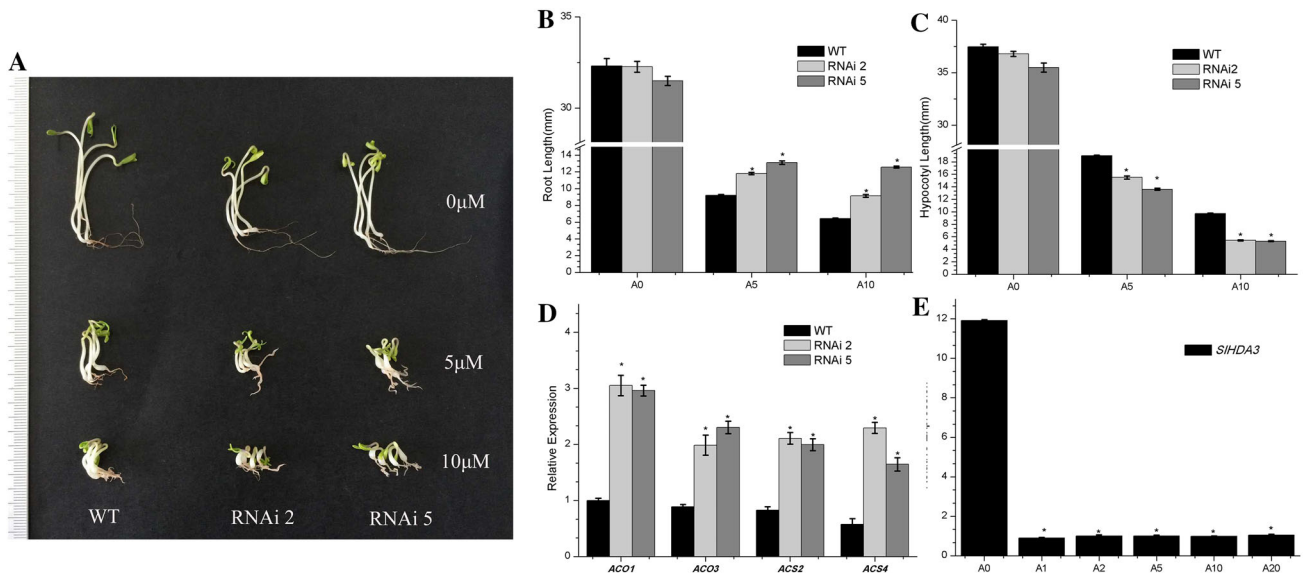


Fig. 5 Ethylene triple response assay. **a** Seedlings of WT and RNAi lines (RNAi 2 and RNAi 5) treated with 0, 5.0 and 10.0 μM ACC. Elongation of root (**b**) and hypocotyl (**c**) growth on different concentrations of ACC. **d** Expression of *ACS2*, *ACS4* and *ACO1*, *ACO3* in seedlings of RNAi lines and the WT. **e** Expression of

SIHDA3 in seedlings of the WT treated with 0 (A0), 1.0 (A1), 2.0 (A2), 5.0 (A5), 10.0 (A10), and 20.0 (A20) μM ACC. Data are the mean $\pm$ SD of three independent experiments. The asterisks indicate statistically significant differences between the WT and transgenic fruits ($P < 0.05$)

Additionally, a fruit-specific lipoxygenase gene, *LOXB*, which is regulated by ethylene (Griffiths et al. 1999); *PG*, a ripening-related cell wall metabolism gene (Giovannoni et al. 1989); and *Pti4*, a defense responses factors, were

assessed. Relative to WT, significant increases of all transcripts were detected in the fruits of transgenic plants (Fig. 6e, f).

SIHDA3 RNAi fruits have a shorter shelf life

WT and transgenic fruits were harvested at B + 7 stage and stored under the same conditions. Twelve days after harvested, the tomato of transgenic lines began to soften, yet those of WT remain hard. Twenty-one days after harvested, transgenic lines tomato were soft and moldy, while WT just began to soften (Fig. 7a).

To figure out the relationship between *SIHDA3* and storability, ripening-related cell wall metabolism genes in B + 4 stage were measured. As shown in Fig. 7b–f, the transcripts of cell wall metabolism genes, *HEX*, *MAN*, *TBG4*, *XTH* and *XYL* were increased significantly in *SIHDA3* RNAi lines fruits.

Discussion

In this study, we studied the histone deacetylase *SIHDA3* gene by analyzing the phenotype, and the expression and metabolites of associated gene in *SIHDA3* RNAi fruit. The results suggested that *SIHDA3* acts as an essential factor involved in the regulation of tomato carotenoid accumulation and the ethylene biosynthesis and plays a negative important role in the fruit ripening.

SIHDA3 influences carotenoid accumulation during tomato fruit ripening

To date, the pathway of carotenoid biosynthesis has been well studied by most ripening-deficient mutant fruits

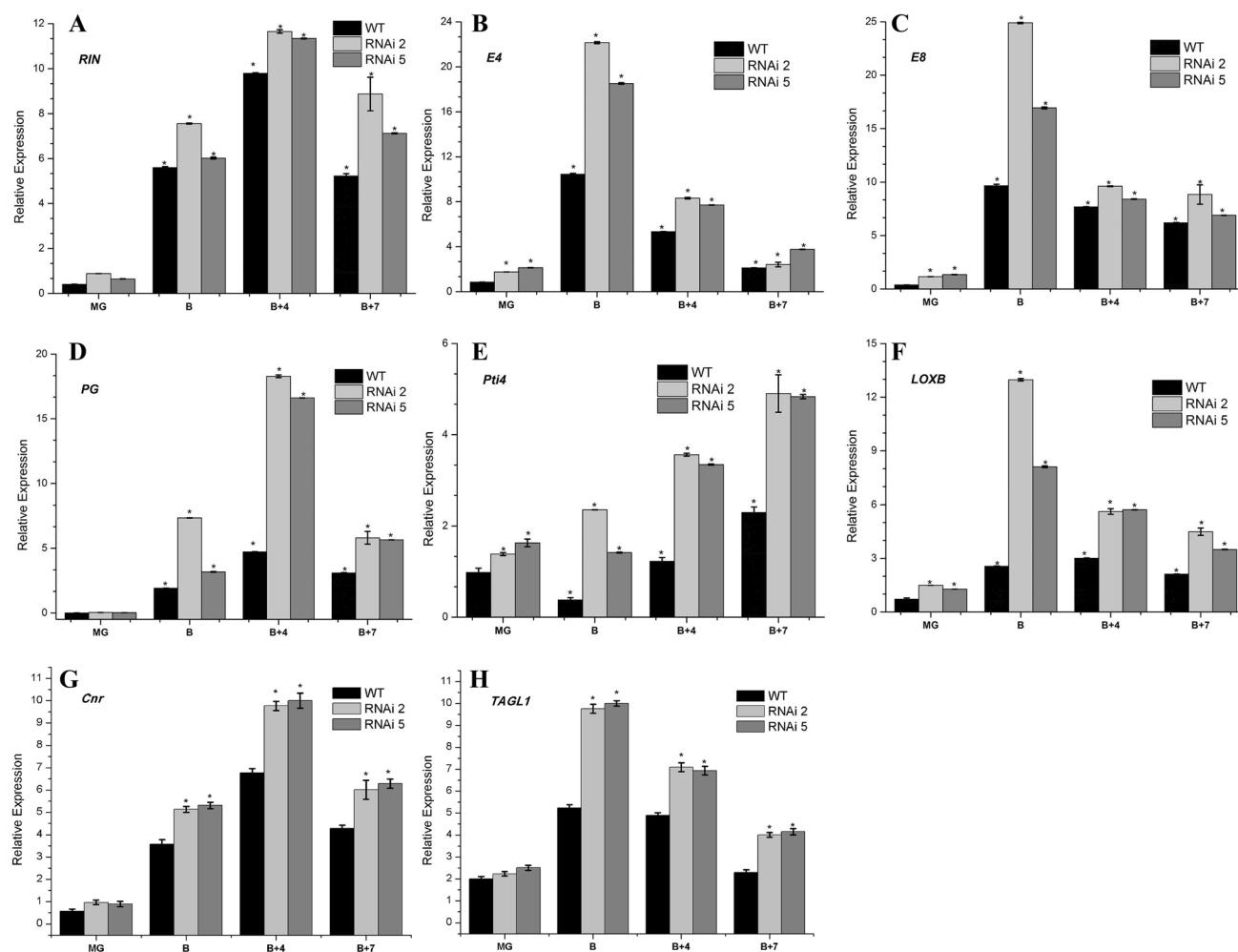


Fig. 6 Ripening-associated gene expression profiles in pericarp between WT and *SIHDA3* RNAi fruits. MG mature green, B breaker, B + 4 4 days after breaker stage, B + 7 7 days after

breaker stage. Data are the mean $\pm$ SD of three independent experiments. The asterisks indicate statistically significant differences between the WT and transgenic fruits ($P < 0.05$)

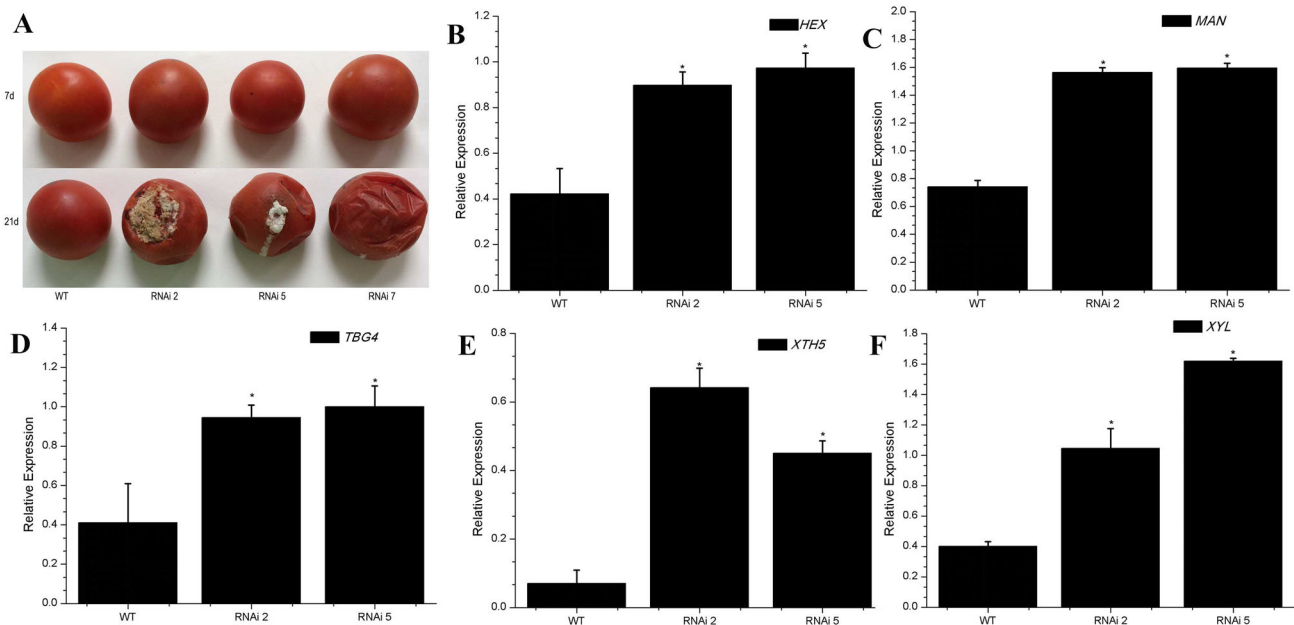


Fig. 7 Phenotype and related genes expression of WT and *SIHDA3* RNAi fruits. **a** Fruits storability phenotype of WT and transgenic lines. 7 and 21 days, post-harvest storage time. **b–f** Relative expression profiles of related genes in the pericarp between WT and *SIHDA3*

RNAi fruits. Data are the mean $\pm$ SD of three independent experiments. The asterisks indicate statistically significant differences between the WT and transgenic fruits ($P < 0.05$)

(Fraser and Bramley 2004). *PSYI*, a critical metabolic flux regulator in the pathway of carotenoid biosynthesis during fruit ripening, is induced by ethylene (Fray and Grierson 1993). It generates a key branching point by the cyclization of lycopene in the carotenoid biosynthetic pathway: one branch leads to β -carotene and its derivative xanthophylls, which is catalyzed by *LCY-B* (the chloroplast-specific lycopene- β -cyclase) and *CYC-B* (the chromoplast-specific lycopene- β -cyclase), whereas the other branch leads to α -carotene and lutein (catalyzed by *LCY-B* and *LCY-E*) (Hirschberg 2001). The content of carotenoid was substantially induced (Fig. 2b) in RNAi lines fruits and explaining why *SIHDA3* RNAi fruits more easily to turn fully red partly. The more red color of pericarp in RNAi fruits than WT implies increased lycopene accumulation and elevated β -carotene. The expression of *PSYI* was significantly up-regulated in pericarp of *SIHDA3* RNAi fruits. On the contrary, the chromoplast and chloroplast lycopene β -cyclases (*CYC-B* and *LCY-B*, *LCY-E*) were down-regulated compared with WT in *SIHDA3* RNAi fruits. The relative ratio of lycopene and β -carotene in ripening tomato fruit is mediated by up-regulation of *PSYI* and down-regulation of *CYC-B*, in which both effects are regulated at least in part by ethylene (Fraser et al. 1994; Ronen et al. 2000; Alba et al. 2005). Similarly, *MADS1*, an MADS box transcription factor negatively regulates ethylene biosynthesis and repression of *MADS1* resulted in induced ethylene and a shift toward lycopene accumulation in ripening fruit (Dong et al. 2013). Inversely, *NAC4*, a

plant-specific NAC transcription factor positively regulates the biosynthesis of ethylene and repressed the expression of *NAC4* resulted in reduced ethylene synthesis and a shift toward the accumulation of β -carotene in ripening fruit (Zhu et al. 2014). According to all previous reports and our study results, we can speculate that *SIHDA3* may act as a negatively factor in regulation of the carotenoid pathway flux toward lycopene and away from β -carotene in *SIHDA3* RNAi fruits.

SIHDA3 as an inhibitor influences ethylene biosynthesis and fruit ripening

In plants, two modes of ethylene synthesis pathways, system 1 and system 2, are well studied and defined (Bleecker and Kende 2000; McMurchie et al. 1972; Barry et al. 2000). System 1 is responsible for providing the basic levels of ethylene in all the plant tissues and functions during normal plant growth and development. System 2 provides a large amount of ethylene at the beginning of fruit ripening. Two key rate-limiting enzymes (ACS and ACO) have been reported in ethylene biosynthesis. And the two key biosynthetic enzymes in the ethylene biosynthesis pathway, ACS, transform SAM to ACC (Adams and Yang 1979) and ACO, and convert ACC to ethylene (Alexander and Grierson 2002).

In this study, the transcript levels of *ACS2*, *ACS4* and *ACO1*, *ACO3* in *SIHDA3* RNAi lines fruits and *SIHDA3* RNAi lines seedlings were detected. The result showed that

the expression of *ACS2*, *ACS4* and *ACO1*, *ACO3* was substantially higher in RNAi transgenic lines than in the WT (Figs. 4b–e, 5d). This result suggests that *SIHDA3* acts as an inhibitor in tomato fruit ripening, probably through function on the ethylene pathway and impacts the ethylene biosynthesis in tomato plant, which was confirmed by the measurement of ethylene content in fruit and the assay of triple response.

Additionally, the length of roots and hypocotyls in RNAi transgenic lines was shorter than that in the WT in the absence of ACC, and the seedlings of RNAi lines were more sensitive to ACC than the WT (Fig. 5a, c), which suggested that more ethylene was produced in the RNAi transgenic plants than that in the WT. These results suggest that *SIHDA3* impacts ethylene biosynthesis both in plant organs and fruits.

Analysis of ripening-related genes expression including the fruit cell wall metabolism-related genes suggests that an induced transcript level of genes was exhibited in pericarp of *SIHDA3* RNAi fruit, including *E4*, *E8*, *PG*, *RIN*, *Pti4*, *Cnr*, *TAGL1* and *LOXB* (Fig. 6a–h), *HEX*, *MAN*, *TBG4*, *XTH5* and *XYL* (Fig. 7b–f). These genes reflect downstream fruit ripening genes activities and further impact carotenoid accumulation, cell wall structure and the production of metabolites (Barry and Giovannoni 2007; Pirrello et al. 2009). Besides, the results of yeast two-hybrid assays and western blots in the previous report also indicated that *TAGL1* interacted with *SIHDA3* regulated gene expression in fruits development in tomato (Zhao et al. 2015). *SIMADS-RIN* and *TAGL1* are essential positive regulators of tomato fruit ripening among the MADS-box proteins and involved in ethylene biosynthesis, ethylene perception and ethylene responsiveness. These results indicate that suppressing the expression of *SIHDA3* promotes ripening-related genes expression and accelerates fruit ripening and softening rate, indicating that *SIHDA3* acts as an inhibitor lies upstream of *SIMADS-RIN* in the fruit ripening regulatory network.

In summary, *SIHDA3* plays an important role in fruit ripening that as a negative regulator by participating in the regulation of hormone ethylene and carotenoid pigmentation. Although the regulation mechanism in the fruit development and ripening remain to be further discovered, *SIHDA3* plays an important role in balancing the activities between positive and negative ripening regulators and adds a new member to regulating fleshy fruit ripening. However, whether the regulation mechanism occurs similarly to other members requires further investigation.

Author contribution statement JG conducted experiments and wrote the manuscript. ZH and XY conceived and designed research. AL and FL analyzed data. ST and YW contributed new reagents and plant materials. GC

revised the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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