Potato Virus X-induced *LeHB-1* Silencing Delays Tomato Fruit Ripening

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ABSTRACT. Tomato (*Solanum lycopersicum*) fruit ripening is a complex genetic trait correlating with notable fruit phenotypic, physiologic, and biochemical changes. Transcription factors (TFs) play crucial roles during this process. *LeHB-1*, an HD-zip homeobox protein, is a ripening-related TF and acts as an important regulator of fruit ripening. However, the detailed biochemical and molecular basis of *LeHB-1* on tomato fruit ripening is unclear. In the current study, the biologic functions of *LeHB-1* were determined by a potato virus X (PVX)-mediated gene-silencing approach. The results indicate that PVX-induced *LeHB-1* silencing in tomato could decrease pigment accumulation and delay fruit ripening. Compared with controls, nonripening flesh retains a greater pH value and a lesser anthocyanin content. By evaluating expression levels of genes related to tomato fruit ripening, we inferred that *LeHB-1* located at the downstream of LeMADS-RIN-mediated regulatory network. In addition, *LeHB-1* silencing mainly disturbed phytoene desaturation and isomerization, and led to a decrease in trans-lycopene accumulation, but did not influence flavonoid biosynthesis directly in tomato fruit. The findings provide a theoretical foundation for illustrating the biologic functions of *LeHB-1* in tomato fruit ripening and quality.

Fruit ripening is an important stage for plants bearing fleshy fruit. During the process of ripening, there are noticeable changes in biophysical and biochemical attributes—such as pigmentation, flesh texture, aroma, and nutrient components—that determine the quality and commodity of fruit (Gapper et al., 2013). Currently, tomato has been an excellent model system for fruit ripening studies as a result of their ease of cultivation, short life cycle, dramatic ripening process, various ripening mutants, efficient gene editing approaches, and fully sequenced genome (Kudo et al., 2017; Tomato Genome Consortium, 2012). Accumulating evidence indicates that the internal determinants of regulating tomato fruit ripening include phytohormone signaling pathways, epigenetic changes, and TF networks (Shinozaki et al., 2018).

As a typical climacteric fruit, perception and signal transduction of ethylene in tomato has been well characterized (Bapat et al., 2010; Cara and Giovannoni, 2008; Wang et al., 2016). Also, it has been well accepted that abscisic acid and indole-3-acetic acid play crucial roles in controlling fruit ripening by a complicated network of feedback and crosstalk among phytohormones (McAttee et al., 2013; Mou et al., 2016; Weng et al., 2015). Some reports suggest that epigenetic modification plays important roles in fruit-ripening progress (Kanazawa et al., 2011; Kasai and Harada, 2015; Manning et al., 2006; Zhong et al., 2013). Liu et al. (2015) defined a direct cause-and-effect relationship between active DNA demethylation and induction of gene expression to explain the correlation between genomic DNA demethylation and fruit ripening.

In addition, a wide range of studies in fruit-specific transcriptional control of ripening in tomato has received considerable

attention (Rohrmann et al., 2012). Several TFs, such as LeMADS-RIN, SIFUL1/SIFUL2, SITAGL1, SIMADS1, SIAP2a, and SINAC4, have been shown to play central regulatory roles by both ethylene-dependent and -independent pathways. Mutations of these TF genes result in phenotypes with changes in all aspects of fruit ripening, including pigment biosynthesis, softening, size and shape, chloroplast degradation, and chromoplast development (Dong et al., 2013; Fujisawa et al., 2013, 2014; Karlova et al., 2011; Vrebalov et al., 2009; Zhu et al., 2014). In intricate transcriptional regulatory networks, *LeHB-1* (GenBank accession no. CAP16664) encodes a 285-aa protein with a conserved homeobox domain (aa 49–125) and a leucine zipper (aa 121–163) (Tomato Genome Consortium, 2012). The protein with a helix-loop-helix-turn-helix structure can bind the promoter of a 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase gene *LeACO1*. *LeHB-1* silencing can result in a reduced *LeACO1* transcript, and thus a considerable delay in tomato fruit ripening. In addition, ectopic overexpression of *LeHB-1* in developing tomato plants can produce multiple flowers within on sepal whorl, which leads to fusion of sepals and petals, and convert sepals into fruit (Lin et al., 2008). However, as a key TF, the detailed biochemical and molecular basis of *LeHB-1* on tomato fruit ripening is unclear. Effects of *LeHB-1* on the biosynthesis of ripening-associated secondary metabolites are also rarely reported.

Virus-induced gene silencing (VIGS) is an attractive reverse-genetics tool for the study of gene function in plants. Upon viral infection, plants use their innate RNAi-mediated defense machinery to target the viral genome explicitly (Senthil-Kumar and Mysore, 2011). PVX, a single-strand RNA virus, is a member of the potexvirus group (Lico et al., 2006). The PVX-mediated VIGS has been used successfully for gene function analysis in tomato fruit (Chen et al., 2015; Lin et al., 2008; Manning et al., 2006; Zhou et al., 2012). Although PVX-mediated VIGS cannot provide uniform efficiency and a stable effect, its advantages are obvious: 1) for keeping away from constructing transgenic lines, PVX-mediated VIGS in tomato fruit is efficient and rapid; 2) the

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induced ripening-related phenotype is distinct and easily identifiable; 3) it can largely simulate targeted gene mutant in transcriptional level; 4) the intensity of PVX-mediated VIGS was various which can be used to assess the function of genes whose mutation is lethal; and 5) partial sequencing information of ripening-related genes is sufficient to silence genes with multiple copies or multiple family members.

In the current study, PVX-induced *LeHB-1* silencing was performed in tomato fruit. Physiologic and biochemical attributes of ripening and nonripening sectors in inoculated fruit were determined first. Subsequently, the expression changes of ripening-associated genes were assessed. Research findings indicated that *LeHB-1* silencing led to a decrease in pigment accumulation and a delay of fruit ripening. In addition, *LeHB-1* could locate at the downstream of LeMADS-RIN-mediated regulatory network and mainly influence carotenoid biosynthesis. These results will provide useful information to explore the functional mechanism of *LeHB-1*.

Materials and Methods

PLANT MATERIALS AND GROWTH CONDITIONS. Tomato cultivar Ailsa Craig (AC) and tobacco (*Nicotiana benthamiana*) were cultured normally in a growth chamber at 25 °C with a photoperiod of 16/8 h day/night. Flowers were tagged at anthesis. Developmental and ripening stages of fruit were recorded as days postanthesis (DPA) and days postbreaker (DPB).

DETECTION OF *LeHB-1* EXPRESSION. Total RNA was extracted from roots, stems, leaves, and fruit (15 DPA, 30 DPA; 5 DPB, 10 DPB) using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). First-strand complementary DNA (cDNA) was produced using the Fast Quant RT Kit (Tiangen, Beijing, China) according to the product manual. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using 2× Ultra SYBR mixture (CWBio, Beijing, China) in a PCR detection system (CFX96; Bio-Rad Laboratories, Hercules, CA). The PCR program was as follows: 95 °C (10 min),

Table 1. Primer pairs used for vector construction and gene expression analysis.

Name	Forward primer (5'-3')	Reverse primer (5'-3')
<i>mLeHB-1</i>	AGTTGAATCGATTAGGGATCTGGGCATATATTT TTCCG	ACTCCACGGCCGTTAAGACCAGAACCATCCAAT AGGC
<i>PVX sequencing</i>	CAGTGTGGCTTGCAAACCTAG	ACTATGGCACGGGCTGTACTAAAG
<i>18S</i>	CGGCTACCACATCCAAGGAAGG	GAGCTGGAATTACCGCGGCTG
<i>LeHB-1</i>	CTACGACGAGCAGTCACCG	GGAACCATAACAGCCACCT
<i>SIMADS1</i>	GTGTAGCTGGATTTCCTACTTCG	GCCGCTGCATTCACCTCAT
<i>SIMYB12</i>	TAACGAGCAATAATGTAGGGAATAG	GTAAGTTAGTGTCAGTATCGTCCCA
<i>SITAGL1</i>	GCAATAACTCCCTGCCTGTA	AGATGAAGAGCCTTGACCC
<i>LeMADS-RIN</i>	AAGGAACCCAACTTCATCAG	TTGTCCCAAATCCTCACCTA
<i>SLAP2a</i>	GGAGTATGAATCCGATGAAGGT	CGATTCCAAATTGTGGTCTT
<i>LeTDR4</i>	ACTGGACTCTCTCACCTTGCGG	AGCTGCACCTTGCTGCTGTGA
<i>LeSPL-CNR</i>	GCTCTCTATCTTCTGTCAATTCCCG	TCGAATACTAGCAGACAGTGCCAAAC
<i>LeNAC-NOR</i>	AGAGAACGATGCATGGAGGTTTGT	ACTGGCTCAGGAAATTGGCAATGG
<i>LeACS1</i>	GTGCTTCAAACAAAGGGAC	GTCCTAACCAAGGCGAAT
<i>LeACS2</i>	GTAGGTGTTGAGAAAAGTGAG	GTCTTAACGAATAATGGTGAGG
<i>LeACS3</i>	CATCTCTGAAAATCAGAAGAGGCT	CCATAAGTCCATTTACGCGTCA
<i>LeACS4</i>	AAATCTCCACCTTCACTAACGAAC	CCTAAGTCCTTGGAAGACTAGACAC
<i>LeACS6</i>	CAATACTGTAGAACAAGGAGC	GGTACTCAGTGAAATAGTCGAC
<i>LeACO1</i>	CACTAACGGGAAGTACAAGAG	CTGCATCACTTCTGGATTGTA
<i>LeACO2</i>	CAACTCCTCAAAGACGGTCG	GTCCCGTCTTTTGTGCGAT
<i>LeACO3</i>	ATGGGACTCGGATGTCCTAGC	CTTCCATAGCCTTCATTGCTTC
<i>LeACO4</i>	CTGTCAACTTAGGTCCAATA	GCTCACTACCAACAACAG
<i>PSY1</i>	AGAGGTGGTGGAAGCAA	TCTCGGGAGTCATTAGCAT
<i>PDS</i>	GGTCACAAACCGATACTGCT	AAACCAGTCTCGTACCAATCTC
<i>ZISO</i>	CCTTCTTCTTCTATACCCGTCG	AGCGTGTGAGCTAAGCACCA
<i>ZDS</i>	AGTGGTTTCTGTCTAAAGGTGG	ACCGAGCACTCATGTTATCAC
<i>CRTISO</i>	AAGACCCACAGACGATACCT	ATCGCCAACACAATATAGACCA
β -CYC	CGACGTGATCATTATCGGAGC	GTGGTGAAGGGTCAACACAACA
β -LCY	TTGACTTAGAACCTCGTTATTGG	AACAGTTCCTTTGTCTATTATCTC
ϵ -LCY	GCCACAGGTTATTAGTCGTCA	CCAGTCCAAATAGGAAAAACGAT
β -CRTR	CTTCTTCTCTACGGTTTCTTCCA	CTCTTATGAACCAGTCCATCGT
<i>CHS</i>	TGGTCACCGTGGAGGAGTATC	GATCGTAGCTGGACCCTCTGC
<i>CHI</i>	GTTTTTCACAAACCAACAGTTCTGAT	GAAGCAGTGCTCGATTCCATAAT
<i>F3H</i>	CACACCGATCCAGGAACCAT	GCCCACCAACTTGGTCTTGTA
<i>F3'H</i>	GCACCACGAATGCACTTGC	CGTTAGTACCGTCGGCGAAT
<i>FLS</i>	GAGCATGAAGTTGGGCCAAT	TGGTGGGTTGGCCTCATTA
<i>DFR</i>	TCCGAAGACGACAACGGTTT	TGACAAGCCAAGAGCCGATAA
<i>ANS</i>	GAAGTAGCACTTGGCGTCGAA	TTGCAAGCCAGGCACCATA
<i>GT</i>	CGAACGACGAAACACTGTTGA	TGCAGCATAGATGGCATTGG
<i>RT</i>	CTGGCAATGCAAACAGAGTGA	TCGACTTGCGGAAGAGTGAGA

followed by 40 cycles at 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 20 s. The intensity change in SYBR Green fluorescence and the threshold cycle (*C_t*) over the background was calculated for each reaction. Samples were normalized using 18S rRNA, and the relative expression levels were measured using the $2^{(-\Delta C_t)}$ analysis method.

CONSTRUCTION OF PVX/*mLeHB-1* VECTOR. A nonsense mutant *LeHB-1* gene (*mLeHB-1*) was designed in which a start codon was replaced by a stop codon. *mLeHB-1* was amplified using DNA polymerase (PrimeSTAR; Takara, Kusatsu, Japan) from fruit cDNA (15 DPA) in a thermal cycler (S1000; Bio-Rad Laboratories). The PCR products were digested by restriction enzymes *Cla* I and *Eag* I (New England BioLabs, Ipswich, MA) and cloned into the PVX vector to generate PVX/*mLeHB-1*. The construct was confirmed by nucleotide sequencing using a pair of PVX sequencing primers. The sequence of primer pairs for vector construction is shown in Table 1.

IN VITRO TRANSCRIPTION AND VIRUS-INDUCED *LEHB-1* SILENCING. The PVX or PVX/*mLeHB-1* vector was linearized using the restriction enzyme *Spe* I (New England BioLabs), purified with a High Pure PCR Product Purification Kit (Roche, Rotkreuz, Switzerland), and then suspended in RNase-free water with 1 unit· μ L⁻¹ RNasin Ribonuclease Inhibitor (Promega, Fitchburg, WI). The Riboprobe In Vitro Transcription System (Promega) was used to synthesize single-strand PVX or PVX/*mLeHB-1* RNA transcripts. Afterward, RNA transcripts were inoculated mechanically on young leaves of *N. benthamiana*. After 10 d of inoculation, systemically infected leaves were collected and freeze-dried in a freeze-drying machine (FreeZone 2.5 Plus; Labconco, Kansas City, MO). Subsequently, ≈ 0.1 g leaf tissue was ground in 2 mL TE buffer (pH 7.5) and needle-injected into the carpel of immature tomato fruit (about 15 DPA). At 5 DPB, fruit with obvious nonripening sectors were photographed, harvested, and stored at -80 °C. The total RNA extraction and first-strand cDNA synthesis of the red zone and green zone of harvested fruit were described as above. The transcripts of PVX/*mLeHB-1* were validated by PCR amplification using a pair of PVX sequencing primers and electrophoresis analysis on a 1% agarose gel (Fig. 1C).

DETERMINATION OF PHYSIOLOGIC AND BIOCHEMICAL PARAMETERS. Soluble solids content was determined using a refractometer (LB20T; Suwei, Shenzhen, China). The pH value was evaluated using a pH meter (3520; Jenway, Stone, UK) in 15 mL diluted juice from 5 g fruit flesh. Anthocyanin was extracted using the method reported by Zhang et al. (2014).

Fig. 1. (A) Developmental expression of *LeHB-1* in tomato cultivar Ailsa Craig and (B–D) construction of potato virus X (PVX)/*mLeHB-1* vector. Fruit development and ripening stages were recorded as days postanthesis (DPA) and days postbreaker (DPB). (B) Analysis of linearized plasmids and in vitro transcription RNA products for PVX and PVX/*mLeHB-1*. (C) Detection of PVX/*mLeHB-1* in tomato fruit at 5 DPB with a biologic repeat by reverse transcription–polymerase chain reaction (RT-PCR) assays. An equal amount of total RNA was used for RT-PCR. PVX/*mLeHB-1G* and PVX/*mLeHB-1R*, respectively, represent green zone and red zone in injected tomato fruit. (D) The phenotype of *Nicotiana benthamiana* inoculated with TE buffer, PVX, or PVX/*mLeHB-1* through mechanical inoculation. Photographs were taken at 10 d postinoculation. Analysis of variance was used to compare more than two means, and Duncan's multiple range tests were used for means separations. Bars represent SD of the means. Lowercase letters indicate significant differences at $P < 0.05$.

Briefly, the sample (5 g) was ground in liquid nitrogen and diluted with methanol supplemented with 0.1 mol·L⁻¹ hydrochloric acid to 30 mL. The suspensions were placed in the dark for 24 h and centrifuged to remove the precipitate. Subsequently, absorbance of the supernatant was measured using a spectrophotometer (SmartSpec Plus, Bio-Rad Laboratories) at 525 nm, and the relative content of anthocyanin was represented by absorbance per gram fresh weight. Chlorophyll content was evaluated as described by Wang et al. (2009). The 5-g sample was ground in liquid nitrogen and diluted with 80% acetone to 30 mL. After adequate mixing and centrifugation, the acetone phase was collected, and absorbance at 645 nm (OD₆₄₅) and at 663 nm (OD₆₆₃) were measured for determination of chlorophyll a and chlorophyll b. Chlorophyll content was calculated using the formula Total chlorophyll content = $8.33 \times (8.02 \times \text{OD}_{663} + 20.20 \times \text{OD}_{645})$, measured in micrograms per gram.

qRT-PCR ANALYSIS. Total RNA extraction and first-strand cDNA synthesis of samples (AC fruit at 30 DPA, AC fruit at 5DPB, the red zone and green zone of AC fruit injected with PVX/*mLeHB-1* at 5 DPB) were described as above. Expression of genes involved in ripening-associated TFs, ethylene biosynthesis, and carotenoid and flavonoid biosynthesis was determined by qRT-PCR using

specific primer pairs as described by Lai et al. (2015), Pandey et al. (2015), and Su et al. (2015). The sequence of all primer pairs for qRT-PCR analysis is shown in Table 1.

STATISTICAL ANALYSIS. Data were collected from at least three independent experiments, and statistical analyses were performed using SPSS (version 20.0; IBM Corp., Armonk, NY). Analysis of variance was used to compare more than two means. Mean separations were analyzed using Duncan's multiple range test. Differences at $P < 0.05$ were considered to be significant.

Results

EXPRESSION PROFILE OF *LeHB-1*. *LeHB-1* can be detected in roots, stems, leaves, flowers, and fruit at different development stages. Of these plant tissues, it has the greatest expressional level in flowers followed by leaves and stems, and little amount in roots. In fruit, the expression level of *LeHB-1* decreased gradually from immature green stage to mature green stage and was followed by a rapid decline at the breaker stage and red ripening stage. The *LeHB-1* transcripts were maintained at a relatively lower level during the fully ripening stage (Fig. 1A).

CONSTRUCTION AND ECTOPIC EXPRESSION OF PVX/*mLeHB-1*. The coding region for *mLeHB-1* was amplified and cloned into the PVX-based vector. RNA transcripts were produced by in vitro transcription (Fig. 1B) and inoculated onto the leaves of *N. benthamiana*. About 10 d after inoculation, virus-inoculated leaves developed obvious chlorotic lesions. In addition, curling and mosaic symptoms were observed on systemically infected leaves (Fig. 1D). The phenotype was consistent with typical symptoms of wild-type PVX infection (Lico et al., 2006). After reverse transcription–polymerase chain reaction (RT-PCR) detection and direct sequencing, the recombinant viral RNAs containing a complete *mLeHB-1* sequence were confirmed in infected *N. benthamiana* leaves.

PVX-INDUCED *LeHB-1* SILENCING. Immature green fruit were needle-injected with leaf sap containing PVX/empty or PVX/*mLeHB-1* RNA transcripts. Under standard greenhouse condition, development of control fruit and inoculated fruit all proceeded normally. About 38 DPA, almost all fruit showed a break in color from green to tannish yellow, pink, or red on no more than 10% of the tomato fruit surface. At 5 DPB, more than 95% of the surface shown red color in control fruit and fruit inoculated with PVX/empty RNA transcripts. However, about 10% of the fruit inoculated with PVX/*mLeHB-1* RNA transcripts shown obvious green or orange sectors that occupied about 20% to 45% of the fruit surface. The maturity of the sarcocarp was heterogeneous as well, as observed in cross-section (Fig. 2). The effectiveness of gene silencing was analyzed at the transcript level by monitoring *LeHB-1* messenger RNA accumulation. The expression of *LeHB-1* in green sectors was downregulated about 30% to 50% than that in red sectors.

EFFECTS OF PVX-MEDIATED *LeHB-1* SILENCING ON FRUIT QUALITY PARAMETERS. Generally, control fruit and fruit inoculated with PVX/empty RNA transcripts possessed similar ripening attributes, which indicates that injection of PVX/empty has no significant influence on fruit ripening. Through PVX-mediated *LeHB-1* silencing, normal ripening (red sector) and ripening delay (green sector) could be inspected visually in one fruit. Soluble solids and anthocyanin contents of the red and green sectors were almost same (Fig. 3A and C). However, pH and chlorophyll content in green sectors were significantly

Fig. 2. Ripening phenotypes of (A) wild-type tomato cultivar Ailsa Craig (AC) fruit, (B) AC fruit injected with potato virus X (PVX), and (C, D) AC fruit injected with PVX/*mLeHB-1* 5 d after breaker. Fruit are bidissected to show internal ripe and/or nonripening tissues.

greater than those in the red sectors (Fig. 3B and D). These results mean that PVX-induced *LeHB-1* silencing was effective and that fruit color correlates positively with physiologic and biochemical changes of fruit during ripening.

EFFECTS OF PVX-MEDIATED *LeHB-1* SILENCING ON EXPRESSION OF GENES RELATED TO FRUIT RIPENING. For most examined genes, their differential expression trends between the green zone and the red zone of *LeHB-1* silenced fruit were similar with those between fruit at 30 DPA and fruit at 5 DPB. For other genes, different or contradictory results may be the result of the different silencing efficiency of *LeHB-1* and individual kinetic trends of genes in developing fruit (Fig. 4).

TFs are required for the initiation and promotion of fruit ripening. In *LeHB-1* silenced fruit, expression levels of *SIMADS1*, *SIMYB12*, and *SIAP2a* were greater in the green sectors than in the red. No statistically significant changes were observed in the expression levels of *SITAGL1*, *LeMADS-RIN*, *LeTDR4*, *LeSPL-CNR*, and *LeNAC-NOR* (Fig. 4A).

As a typical respiration climacteric fruit, ethylene biosynthesis is an important parameter for tomato fruit ripening. Comparing red sectors, the expression levels of *LeACS1*, *LeACS4*, *LeACO1*, and *LeACO3* increased significantly; the expressions of *LeACS6* decreased significantly in the green sectors of *LeHB-1* silenced fruit. No statistically significant

Fig. 3. Effect of potato virus X (PVX)-induced *LeHB-1* silencing on (A) soluble solids content, (B) pH, (C) anthocyanin content, and (D) chlorophyll content in tomato cultivar Ailsa Craig (AC) fruit at 5 d postbreaker. mLeHB-1R and mLeHB-1G represent the red zone and green zone, respectively, of AC fruit injected with PVX/*mLeHB-1*. Analysis of variance was used to compare more than two means, and Duncan's multiple range tests were used for means separations. Lowercase letters indicate significant differences at $P < 0.05$; bars represent sd.

changes were observed in the expression levels of *LeACS2*, *LeACS3*, *LeACO2*, and *LeACO4* (Fig. 4B).

The color change from green to red is a very important indicator of tomato ripening and can be measured easily by chromametry. This change is associated with the degradation of chlorophylls and the composition of carotenoid. The levels of transcripts coding for carotenoid biosynthesis enzymes were monitored in *LeHB-1* silenced fruit. Results indicate that, compared with the red sectors, the expression of *SIZISO*, *SIZDS*, *CRTISO*, and β -*CRTR* was downregulated in the green sectors. The expression of other genes related to carotenoid biosynthesis in the current study were not influenced by *LeHB-1* silencing (Fig. 4C).

The flavonoid group as an important secondary metabolite plays crucial roles in the tomato development and ripening process. The expressions of genes involved in flavonoid synthesis in *LeHB-1* silenced fruit was determined as well. The data indicate that almost all genes showed greater expression levels in green sectors than in red (Fig. 4D).

Discussion

In this study, PVX-mediated *LeHB-1* silencing was performed to estimate the function of *LeHB-1* on tomato fruit ripening. When the PVX vector that harbored a host-derived target gene sequence (*mLeHB-1*) infected immature fruit, a double-strand RNA copy of the insert was produced and induced the activation of the host plant's RNA silencing pathway, resulting in the subsequent degradation of the endogenous *LeHB-1* transcript (Becker and Lange, 2010; Senthil-Kumar and Mysore, 2011). Hence, fruit failed to ripen normally and generated a distinct green or orange sector. Compared with mature parts, the nonripening parts had a greater pH value and chlorophyll content, which corresponds to the

immature phenotype. The soluble solids content and anthocyanin content were not influenced by *LeHB-1* silencing.

Transcriptional profiling studies indicate that amounts of TFs show expression alterations during fruit development and ripening consistent with functions (Arhondakis et al., 2016). Mutations of TF genes, including *LeMADS-RIN*, *LeSPL-CNR*, and *LeNAC-NOR*, can abolish the normal ripening process. In addition, *SITAGL1*, *TDR4*, *SIAP2a*, *SIMADS1*, and *SIMYB12* have been found to play significant roles in tomato fruit ripening. Among them, *LeMADS-RIN* was considered to be a master regulator because its target genes are involved in ethylene synthesis and signaling, cell wall modification, carotenoid accumulation, aroma formation, and transcriptional regulation of ripening-related TF genes (Fujisawa et al., 2013). Meanwhile, *LeMADS-RIN*, *SIFUL1/SIFUL2*, and *SITAGL1* could form a higher order tetramer

that is mainly responsible for ripening regulation (Fujisawa et al., 2014; Shima et al., 2013). Several studies using a chromatin immunoprecipitating approach revealed that *LeMADS-RIN* interacted directly with the promoters of *SIAP2a*, *LeTDR4*, *LeNAC-NOR*, *LeSPL-CNR*, and *LeHB-1* (Fujisawa et al., 2012, 2013; Ito et al., 2008; Qin et al., 2012). *LeHB-1* was the only reported negatively regulated target (Martel et al., 2011). In the current study, expression levels of these TFs were not influenced in *LeHB-1* silenced fruit, which is consistent with reports that the promoter of *LeHB-1* was the target of *LeMADS-RIN*, and also indicates that *LeHB-1* is located downstream from the *LeMADS-RIN*-mediated regulatory network. *SIMADS1* belongs to the MADS-box gene family and plays an important role in fruit ripening as a repressive modulator (Dong et al., 2013). Its transcripts decreased significantly in accordance with fruit ripening. As a result of ripening delay in the *LeHB-1* silencing sector, the greater expression level of *SIMADS1* in green sectors was reasonable.

In climacteric fruit, ethylene is present throughout the various development stages and is essential for the onset and completion of the ripening process (Bapat et al., 2010). Two systems of ethylene biosynthesis have been studied extensively in plants. System 1, with an autoinhibitory manner, represents basal ethylene in vegetable tissues and immature fruit. System 2, with an autocatalytic manner, stands for a significant increase in ethylene production involved in flower senescence and fruit ripening. The main rate-limiting step in the ethylene biosynthetic pathway is the production of ACC. The reaction is catalyzed by ACC synthase (ACS), which is followed by the conversion of ACC to ethylene by ACC oxidase (ACO). System 2 regulated mainly by *LeACS2*, *LeACS4*, and *LeACS6*. *LeACS2* and *LeACS4* are controlled in a positive feedback manner, whereas *LeACS6* is regulated by a negative feedback

Fig. 4. Expression of genes related to (A) ripening-associated transcription factors, (B) ethylene biosynthesis, (C) carotenoid biosynthesis, and (D) flavonoid biosynthesis in tomato cultivar Ailsa Craig (AC) fruit injected with potato virus X (PVX)/*mLeHB-1*. The 5 DPB and 30 DPA represent fruit at 5 d postbreaker and fruit at 30 d postanthesis, respectively. *mLeHB-1R* and *mLeHB-1G* represent the red zone and green zone of AC fruit, respectively, injected with PVX/*mLeHB-1* at 5 DPB. Analysis of variance was used to compare more than two means, and Duncan's multiple range tests were used for means separations. Different lowercase letters represent significant differences at $P < 0.05$ in the expression of indicated genes among four groups; bars represent sd. ACS, 1-aminocyclopropane-1-carboxylate (ACC) synthase; ACO, ACC oxidase; PSY, phytoene synthase; PDS, phytoene desaturase; ZISO, zeta-carotene isomerase; ZDS, zeta-carotene desaturase; CRTISO, carotenoid isomerase; β -CYC, β -cyclase; β -LCY, lycopene β -cyclase; ϵ -LCY, lycopene ϵ -cyclase; β -CRTR, β -carotene hydroxylase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavanol 4-reductase; ANS, anthocyanidine synthase; GT, glucosyltransferase; RT, rhamnosyltransferase.

system. Expression of *LeACS2* and *LeACS4* peak and *LeACS6* sustains a lower level at around breaker stage. Five genes encoding the ACO enzyme have been defined in tomato and three of them—*LeACO1*, *LeACO3*, and *LeACO4*—have been shown to be expressed differentially in fruit. The expression levels of *LeACO1* and *LeACO4* increase dramatically at the onset of climacteric burst and ripening. The expression of *LeACO3* is reduced but transitory at the breaker stage (Cara and Giovannoni, 2008). In the current study, the expression of

LeACS2, *LeACS4*, *LeACO1*, *LeACO3*, and *LeACO4* is upregulated in the green sector, whereas the expression of *LeACS6* is downregulated. Compared with the red sector, the green sector was at around the breaker stage, and the respiration climacteric in the green sector was only beginning. The ripening of tomato was delayed considerably by *mLeHB-1* silencing.

The red color of ripening tomato fruit is mainly a result of carotenoid (in flesh) and flavonoid (in peel) pigments (Ballester et al., 2010). The carotenoid biosynthetic pathway in tomato has been well described by Giuliano (2014). Briefly, two molecules of geranylgeranyl diphosphate are first condensed to 15-cis-phytoene by phytoene synthases. The 15-cis-phytoene is then transformed into all-trans-lycopene through a series of reactions of phytoene desaturase (PDS), zeta-carotene desaturase (ZDS), carotenoid isomerase (CRTISO), and zeta-carotene isomerase (ZISO). Subsequently, the conversion of lycopene to γ -carotene and δ -carotene are catalyzed by lycopene β -cyclases (β -CYC) and ϵ -cyclase (ϵ -LCY). The β -carotene and α -carotene are further synthesized by β -CYC. Finally, the carotenes are transformed into violaxanthin and lutein by β -carotene hydroxylases (β -CRTR) (Su et al., 2015). In this study, expressional intensities of *PSY1*, *PDS*, ϵ -LCY, β -LCY, and β -CYC had no significant differences between the red and green sectors of tomato fruit. However, the expression of *ZISO*, *ZDS*, and *CRTISO* was downregulated in nonripening zones. This indicates the desaturation and isomerization of phytoene were disturbed by *LeHB-1* silencing, which then led to a decrease in all-trans-lycopene accumulation.

Besides carotenoids, flavonoids are essential for determining the color of tomato fruit peel (Ferreya et al., 2012). They are rarely accumulated in the fruit flesh because of a lack of expression of biosynthetic genes. The first committed step of flavonoid biosynthesis is the condensation of one molecule of coumaroyl CoA and three molecules of malonyl CoA to form naringenin-chalcone in a reaction catalyzed by chalcone synthase. Naringenin is then generated by chalcone isomerase. Subsequently, naringenin is hydroxylated to the precursor of delphinidin, pelargonidin, or cyanidin by flavonoid 3-hydroxylase, flavonoid 3'-hydroxylase, and/or flavonoid 3'5'-hydroxylase (F3'5'H).

Finally, under catalyzing of dihydroflavonol reductase and anthocyanidine synthase, the anthocyanin pigments are produced; under catalyzing of flavonol synthase, myricetin, kaempferol, and quercetin are produced. In addition, quercetin can be transformed to rutin in a series of actions catalyzed by glucosyltransferase and rhamnosyltransferase (Koes et al., 2005). The genes of these key enzymes almost show the same pattern of expression in tomato peel. Levels of transcripts increase with fruit development, peak at the breaker or turning stage, and decline gradually in red-stage fruit (Pandey et al., 2015). In the current study, all the examined genes related to flavonoid biosynthesis were upregulated in the green sector 5 DPB, which is similar to the expression trend of TF gene *LeMYB12*. The result is consistent with a report that *LeMYB12* plays an important role in regulating the flavonoid pathway (Ballester et al., 2010). The upregulation of primary genes in flavonoid biosynthesis pathway may be due to the delayed respiratory climacteric induced by *LeHB-1* silencing. Therefore, we speculate that *LeHB-1* does not affect flavonoid biosynthesis in a direct way.

In conclusion, PVX-induced *LeHB-1* silencing in tomato can decrease pigment accumulation and delay fruit ripening. Through qRT-PCR analysis, we inferred that *LeHB-1* is located downstream from the *LeMADS-RIN*-mediated regulatory network and it did not influence flavonoid biosynthesis directly. In addition, *LeHB-1* can influence carotenoid biosynthesis by regulating the desaturation and isomerization of phytoene. Further exploration is needed to illuminate the exact mechanism of *LeHB-1* in regulating tomato fruit ripening.

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