Molecules and Cells

Silencing of the Target of Rapamycin Complex Genes Stimulates Tomato Fruit Ripening

Ilyeong Choi[1,6], Chang Sook Ahn[1,2,6], Du-Hwa Lee[1,3,6], Seung-A Baek[4], Jung Won Jung[4], Jae Kwang Kim[4], Ho-Seok Lee[5], and Hyun-Sook Pai[1,*]

[1]Department of Systems Biology, Yonsei University, Seoul 03722, Korea, [2]Platform Technology Research Center, Corporate R&D, LG Chem/LG Science Park, Seoul 07796, Korea, [3]Gregor Mendel Institute (GMI), Austrian Academy of Sciences, Vienna BioCenter (VBC), 1030 Vienna, Austria, [4]Division of Life Sciences, College of Life Sciences and Bioengineering, Incheon National University, Incheon 22012, Korea, [5]Department of Biology, Kyung Hee University, Seoul 02447, Korea, [6]These authors contributed equally to this work.
*Correspondence: hspai@yonsei.ac.kr
https://doi.org/10.14348/molcells.2022.2025
www.molcells.org

The target of rapamycin complex (TORC) plays a key role in plant cell growth and survival by regulating the gene expression and metabolism according to environmental information. TORC activates transcription, mRNA translation, and anabolic processes under favorable conditions, thereby promoting plant growth and development. Tomato fruit ripening is a complex developmental process promoted by ethylene and specific transcription factors. TORC is known to modulate leaf senescence in tomato. In this study, we investigated the function of TORC in tomato fruit ripening using virus-induced gene silencing (VIGS) of the TORC genes, *TOR*, lethal with SEC13 protein 8 (*LST8*), and regulatory-associated protein of TOR (*RAPTOR*). Quantitative reverse transcription-polymerase chain reaction showed that the expression levels of tomato TORC genes were the highest in the orange stage during fruit development in Micro-Tom tomato. VIGS of these TORC genes using stage 2 tomato accelerated fruit ripening with premature orange/red coloring and decreased fruit growth, when control tobacco rattle virus 2 (TRV2)-myc fruits reached the mature green stage. TORC-deficient fruits showed early accumulation of carotenoid lycopene and reduced cellulose deposition in pericarp cell walls. The early ripening fruits had higher levels of transcripts related to fruit ripening transcription factors, ethylene biosynthesis, carotenoid synthesis, and cell wall modification. Finally, the early ripening phenotype in Micro-Tom tomato was reproduced in the commercial cultivar Moneymaker tomato by VIGS of the TORC genes. Collectively, these results demonstrate that TORC plays an important role in tomato fruit ripening by modulating the transcription of various ripening-related genes.

Keywords: carotenoid biosynthesis, fruit ripening, target of rapamycin complex, tomato, virus-induced gene silencing

INTRODUCTION

Tomato (*Solanum lycopersicum*) is a commercially important crop that provides a complex set of phytochemicals, vitamins, and minerals for the human diet. Extensive research has been done to understand the regulatory mechanisms of fruit development and ripening in tomato. As a climacteric fruit, tomato absolutely requires ethylene to ripen and characteristically shows marked increases in respiration rate and ethylene production at the onset of ripening (Giovannoni, 2007; Klee and Giovannoni, 2011). These events are followed by fruit color changes (due to carotenoid accumulation), softening, and

Received 16 November, 2021; revised 19 April, 2022; accepted 25 April, 2022; published online 22 August, 2022

eISSN: 0219-1032

the accumulation of aromas, flavors, and soluble sugars, all of which indicate maturity of the fruit. The complex network of hormones and transcription factors (TFs) coordinates the fruit ripening process by controlling the transcription of many ripening genes and their post-transcriptional events (Quinet et al., 2019).

Ethylene is important for both the initiation and progression of fruit ripening in tomato (Klee, 2002). Moreover, 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) and ACC oxidase (ACO) are the key enzymes in ethylene biosynthesis; ACS converts S-adenosylmethionine to ACC, which is subsequently converted to ethylene by ACO (Klee and Giovannoni, 2011). ACS is the rate-limiting enzyme for ethylene production during tomato ripening (Liu et al., 2015; Sharma et al., 2021). The tomato genome has fourteen ACS- and six ACO-encoding genes, and the expression of some of these genes is regulated in a tissue- and developmental stage-specific manner (Houben and Van de Poel, 2019; Liu et al., 2021; Yokotani et al., 2009). Typically, low concentrations of ethylene are produced at basal levels (system-1) in all tissues during vegetative growth, while a major burst of auto-stimulatory ethylene synthesis (system-2) occurs during the ripening of tomato fruits (Alexander and Grierson, 2002; Barry et al., 2000). In addition to temporal synthesis, ethylene perception and response through the ethylene signaling pathway are essential for the initiation and completion of fruit ripening (Liu et al., 2015).

Transcriptome analyses have shown that massive transcriptional reprogramming occurs during tomato fruit ripening (Gapper et al., 2013; Kumar et al., 2014; Stanley and Yuan, 2019). The MADS-box TF ripening inhibitor (RIN; MADS-RIN) is known to play a critical role in tomato fruit ripening. Recent evidence suggests that RIN regulates the transcription of numerous ripening-related genes by forming a ripening quartet with other MADS-box proteins, such as tomato agamous-like 1 (TAGL1), fruitfull 1 (FUL1), and FUL2, on the ripening-gene promoters (Bemer et al., 2012; Ito et al., 2020; Vrebalov et al., 2009). RIN is thought to function upstream of ethylene, but recent studies have shown that ethylene can initiate ripening in a RIN-independent manner, and RIN can also act ethylene-independently in some ripening processes (Li et al., 2020). Different classes of TFs, such as non-ripening (NOR; NAC-NOR) and colorless non-ripening (Cnr; the squamosa-box binding protein), are also important for tomato fruit ripening; both the *nor* and *cnr* mutants do not accumulate lycopene or soften (Eriksson et al., 2004; Giovannoni et al., 2017; Manning et al., 2006; Wang et al., 2020). These TFs collectively modulate the transcription of numerous ripening-related genes that are involved in carotenoid biosynthesis, cell wall metabolism, and accumulation of sugars and volatile compounds (Adaskaveg et al., 2021; Fujisawa et al., 2013; 2014; Li et al., 2021). Taken together, these results suggest that ethylene, RIN, and other TFs are required for the initiation and completion of tomato fruit ripening.

The evolutionarily conserved target of rapamycin (TOR) protein kinase is a central regulatory hub that controls the cell growth and metabolism according to cellular environmental information, such as nutrient availability, energy status, and environmental stresses (Bögre et al., 2013; Dobrenel et al., 2016; Wullschleger et al., 2006; Xiong and Sheen, 2013). When activated, TOR stimulates anabolic processes, such as mRNA transcription and translation, under favorable conditions, while its inactivation results in the induction of autophagy, senescence, and stress responses under unfavorable conditions. The plant TOR complex (TORC) consists of TOR kinase, regulatory-associated protein of TOR (RAPTOR), and lethal with SEC13 protein 8 (LST8) (Dobrenel et al., 2016; Ryabova et al., 2019). Null mutations in *Arabidopsis TOR* causes embryo lethality, while reduced expression of the gene results in growth retardation and early senescence, suggesting that TOR is essential for plant growth and development (Deprost et al., 2007; Menand et al., 2002). There are two *RAPTOR* genes (*RAPTOR1A* and *RAPTOR1B*) and two *LST8* genes (*LST8-1* and *LST8-2*) in *Arabidopsis*, but only the *RAPTOR1B* and *LST8-1* mutants showed significant growth defects (Anderson et al., 2005; Moreau et al., 2012; Salem et al., 2017). Thus, RAPTOR and LST8 appear to be important, but not essential, for plant vegetative growth, as observed in animals and yeast.

Inhibition of TOR kinase by chemical inhibitors, such as KU63794, AZD8055, and Torin1, causes severe growth retardation in Micro-Tom tomato seedlings (Xiong et al., 2016). In *Arabidopsis* seedlings, TOR inactivation by AZD8055 causes accumulation of ACS2 and ACS6, isozymes of rate-limiting and labile ACS, for ethylene biosynthesis, suggesting that TOR regulates ACS protein levels (Zhuo et al., 2020). These results suggest that active TOR represses ethylene biosynthesis and senescence in the vegetative tissues of *Arabidopsis* and tomato plants. Tomato fruits display senescence symptoms and cell death after ripening (Kaulfürst-Soboll et al., 2021; Lai et al., 2020); thus, ripening can be considered as the first step of senescence processes. It is an interesting question if TOR regulates tomato fruit ripening as it modulates senescence in vegetative tissues. In this study, we investigated the functions of TORC in tomato fruit ripening by virus-induced gene silencing (VIGS). VIGS of *TOR*, *RAPTOR*, and *LST8* in tomato resulted in an orange/red color change in fruits in the mature green stage, when tobacco rattle virus 2 (TRV2)-myc control fruits were still green in color. These color changes were accompanied by increased transcript levels of ripening-related TFs, ethylene biosynthesis, carotenoid biosynthesis, and cell wall metabolism genes. Accordingly, the early ripened tomato contained high levels of lycopene carotenoids and showed a reduction in cellulose staining in pericarp cell walls, compared with TRV2-myc control. Taken together, these results suggest that TORC plays a critical role in tomato fruit ripening by modulating the transcription of ripening-related genes.

MATERIALS AND METHODS

Plant materials and growth conditions

Tomato plants (*S. lycopersicum* cv. Micro-Tom and cv. Moneymaker) were grown in a growth chamber at 22°C under a 16-h/8-h light:dark cycle with a light intensity of 100-150 μmol m^{-2}·s^{-1}.

VIGS in tomato fruits

VIGS was performed in tomato fruits using TRV2 vectors

(Burch-Smith et al., 2006), as previously described (Fu et al., 2005). TRV2 vectors containing 339-bp, 400-bp, and 336-bp cDNA fragments of the tomato *S. lycopersicum TOR* (*SlTOR*), *S. lycopersicum LST8* (*SlLST8*), and *S. lycopersicum RAPTOR* (*SlRAPTOR*) genes, respectively, were transformed into *Agrobacterium tumefaciens GV3101* strains. *A. tumefaciens GV3101* strains containing TRV1 (pBINTRA) and recombinant TRV2 vectors were grown overnight at 28°C in Luria-Bertani medium containing 10 mM MES (MES-KOH, pH 5.7), 20 μM acetosyringone, kanamycin (50 μg/ml), and rifampicin (50 μg/ml). The *Agrobacterium* cells were then harvested and resuspended in infiltration medium (10 mM $MgCl_2$, 10 mM MES-KOH, pH 5.7, and 200 μM acetosyringone). TRV1 and TRV2 infiltration media were mixed in the ratio of 1:1 at OD_{600} = 1, as previously described (Oh and Kim, 2021), and slowly infiltrated into the space between pedicel and pericarp of stage 2 tomato fruits. Phenotypes were observed 13-24 days after infiltration (DAI). The fruit stages were arranged by size, color, and days post-anthesis (DPA).

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

For RNA extraction, tomato fruits were harvested from VIGS plants at 14, 20, or 23 DAI, and homogenized by mortar and pestle in liquid nitrogen. Total RNA was extracted using the IQeasy Plus Plant RNA Extraction Mini Kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions. One thousand micrograms of total RNA was used for cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). qRT-PCR was performed with diluted cDNAs (1:40) using SYBR Premix EX Taq (Nanohelix, Korea) and StepOnePlus Real-Time PCR system, as previously described (Lee et al., 2017). *ACTIN2* mRNA was used as a control for normalization.

Materials for high-performance liquid chromatography (HPLC)

Lycopene (HPLC grade, ≥ 95%), 13-cis-β-carotene (HPLC grade, ≥ 96%), all-trans-β-carotene (HPLC grade, ≥ 96%), 9-cis-β-carotene (HPLC grade, ≥ 99%), lutein (HPLC grade, ≥ 96%), and β-apo-8'-carotenal (HPLC grade, ≥ 97%) were purchased from CaroteNature (Lupsingen, Switzerland). Potassium hydroxide and ammonium acetate (molecular biology grade) were purchased from Sigma-Aldrich (USA). Methanol (HPLC grade) and methyl tert-butyl ether (HPLC grade) were purchased from Daejung Chemical & Metal (Korea) and Fisher Scientific (USA), respectively. Dichloromethane (HPLC grade) was purchased from Burdick & Jackson (USA). Water was produced using a Millipore water purification system (Milli-Q Direct 8; Millipore, USA).

HPLC for analyses of carotenoids

The pericarp tissues of tomato fruits were chopped into fine pieces and frozen in liquid nitrogen. The frozen samples were ground to a fine powder using a mortar and pestle and lyophilized. Carotenoids were extracted from the lyophilized tomato fruit samples and analyzed using HPLC (Agilent, France) as previously described (Baek et al., 2019). Quantification of carotenoids was performed as described by Cucu et al. (2012). Each carotenoid was identified by the retention time, compared with those of the standard carotenoids. Carotenoid contents were calculated based on the peak areas using the standard calibration curve of each carotenoid compound. The β-apo-8'-carotenal was used as an internal control for HPLC.

Confocal microscopy

Protoplasts were prepared from tomato pericarp tissues after VIGS. The autofluorescence of carotenoids and chlorophylls was observed using a confocal laser scanning microscope (Zeiss LSM510; Zeiss, Germany) at 650-750 nm and 500-600 nm for chlorophyll *a/b* and carotenoids, respectively, as previously described (Yazdani et al., 2019).

Total chlorophyll contents

Chlorophyll was extracted from pericarps of VIGS tomato fruits by using 80% acetone solution. The total chlorophyll content (mg/g fresh weight) was calculated as previously described (Nath et al., 2011). The experiments were performed three times for statistical analyses.

Statistical analysis

GraphPad PRISM 9.0 (GraphPad Software, USA) was used for statistical assessment. Unless indicated otherwise, the statistical analyses were performed by comparison between TRV2-myc and other VIGS tomato fruits. For the single comparison, the statistical significance of the differences was calculated by the Student's *t*-test. For the multiple comparisons, the statistical significance of the differences was calculated by one-way ANOVA followed by Dunnett's multiple comparisons test. The asterisks indicate the statistical significance from the calculated *P* values ($*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$).

RESULTS

Expression patterns of TORC genes in plant organs and fruit development stages

The tomato genome has a single copy of *TOR* (Solyc01g106770) and *LST8* (Solyc03g059310), designated *SlTOR* and *SlLST8*, respectively. There are two *RAPTOR* genes in tomato, Solyc09g014780 and Solyc10g076260, and the DNA sequences of these two genes were very similar to each other. For the analysis of *RAPTOR* in tomato, we used the Solyc09g014780 sequence, designated *SlRAPTOR*. We first examined transcript levels of TORC component genes of tomato, *SlTOR*, *SlLST8*, and *SlRAPTOR*, using qRT-PCR in different organs and fruit development stages of Micro-Tom tomato. qRT-PCR was performed with gene-specific primers (Supplementary Table S1), using mRNAs extracted from mature leaves, flowers, and different fruit stages (Musseau et al., 2017): stage 1 (5 DPA), stage 2 (10 DPA), mature green, breaker, orange, and red (Fig. 1A). Transcript levels of *SlTOR*, *SlLST8*, and *SlRAPTOR* in flowers and fruits were shown relative to those in the leaf. All three genes were highly expressed in flowers compared to leaves. The TORC transcript levels in fruits were relatively low in the stage 1, stage 2, mature green stage, and breaker stage, but significantly increased in the orange stage, followed by a decrease in the red stage

(Fig. 1B). The correlated induction of *SlTOR*, *SlLST8*, and *SlRAPTOR* in the orange stage indicates that TORC may play a role in tomato fruit ripening.

VIGS of *SlTOR*, *SlLST8*, and *SlRAPTOR* in tomato fruits

VIGS was performed to silence *SlTOR*, *SlLST8* and *SlRAPTOR* genes in fruits of *S. lycopersicum* cv. Micro-Tom. The *SlTOR*, *SlLST8*, and *SlRAPTOR* cDNA fragments containing 399-bp, 400-bp, and 336-bp coding regions were cloned into the TRV2 vector to generate TRV2-SlTOR, TRV2-SlLST8 and TRV2-SlRAPTOR, respectively, which were then transformed into *Agrobacterium* as described in Materials and Methods section (Fig. 2A). TRV2-myc was used as the control. VIGS was performed by slowly infiltrating the *Agrobacterium* cells into the space between the pedicel and pericarp of stage 2 tomato fruits. Twenty DAI, VIGS of *SlTOR*, *SlLST8*, and *SlRAPTOR* slightly inhibited tomato fruit growth, but strongly induced premature fruit color change from green to orange/red, while TRV2-myc control fruit grew larger and maintained a green color (Fig. 2B). Furthermore, the placenta and locular

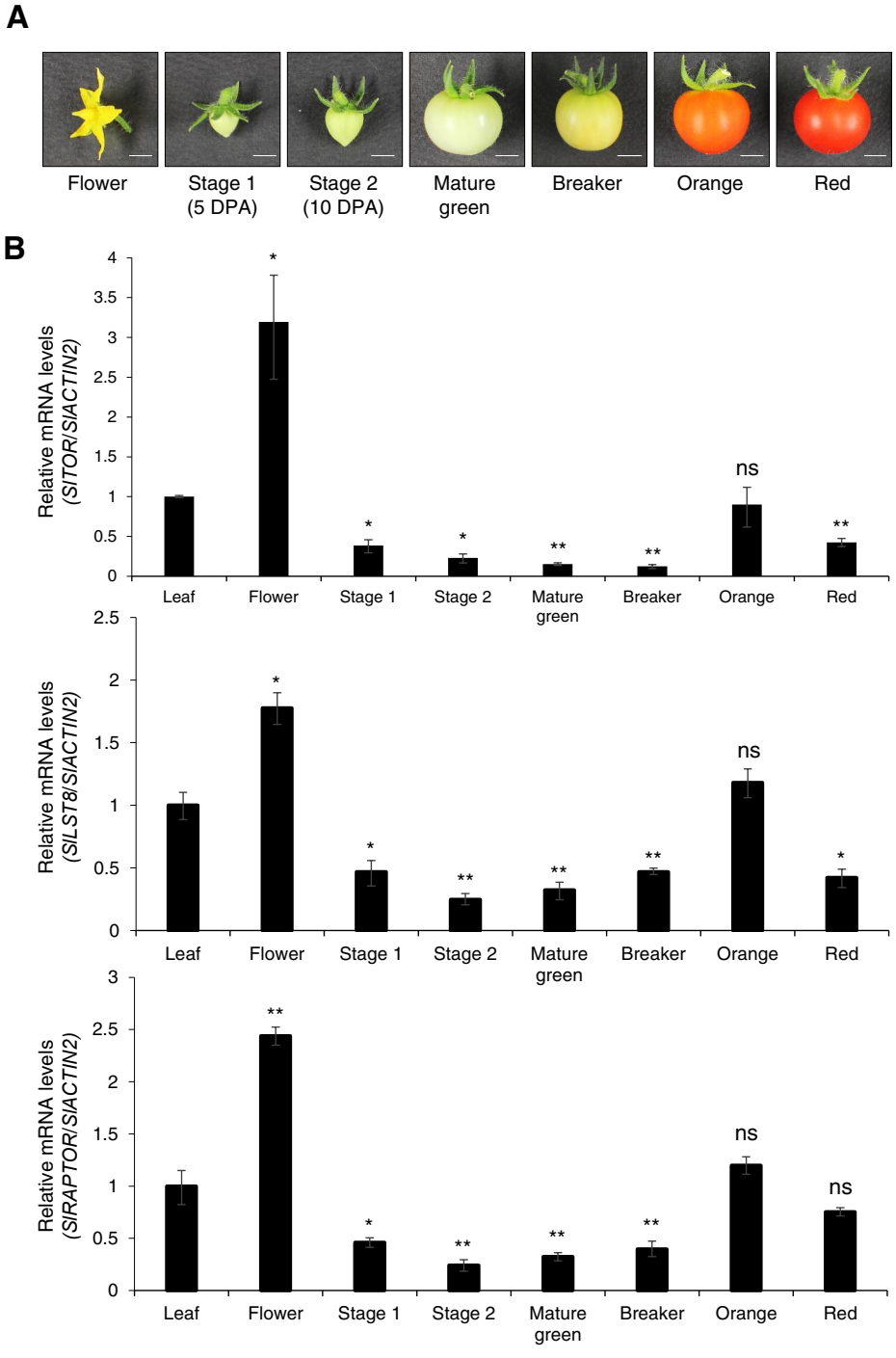

Fig. 1. Gene expression profiles of the TOR complex (TORC) genes in tomato fruits at different developmental stages. (A) Growth characteristics of Micro-Tom tomato fruits at different developmental stages. DPA, days post-anthesis. Scale bars = 0.5 cm. (B) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses. Total RNA was isolated from the leaves, flowers, and fruit pericarps at different developmental stages. The transcript levels of *S. lycopersicum TOR* (*SlTOR*), *S. lycopersicum* lethal with SEC13 protein 8 (*SlLST8*), and *S. lycopersicum* regulatory-associated protein of TOR (*SlRAPTOR*) were normalized by *ACTIN2* mRNA levels, and expressed relative to those in the leaf. Statistical significance was analyzed for the differences in TORC transcript levels between the leaf and other tissues. Data represent the mean ± SE of three replicates per experiment ($*P \leq 0.05$; $**P \leq 0.01$; ns, not significant).

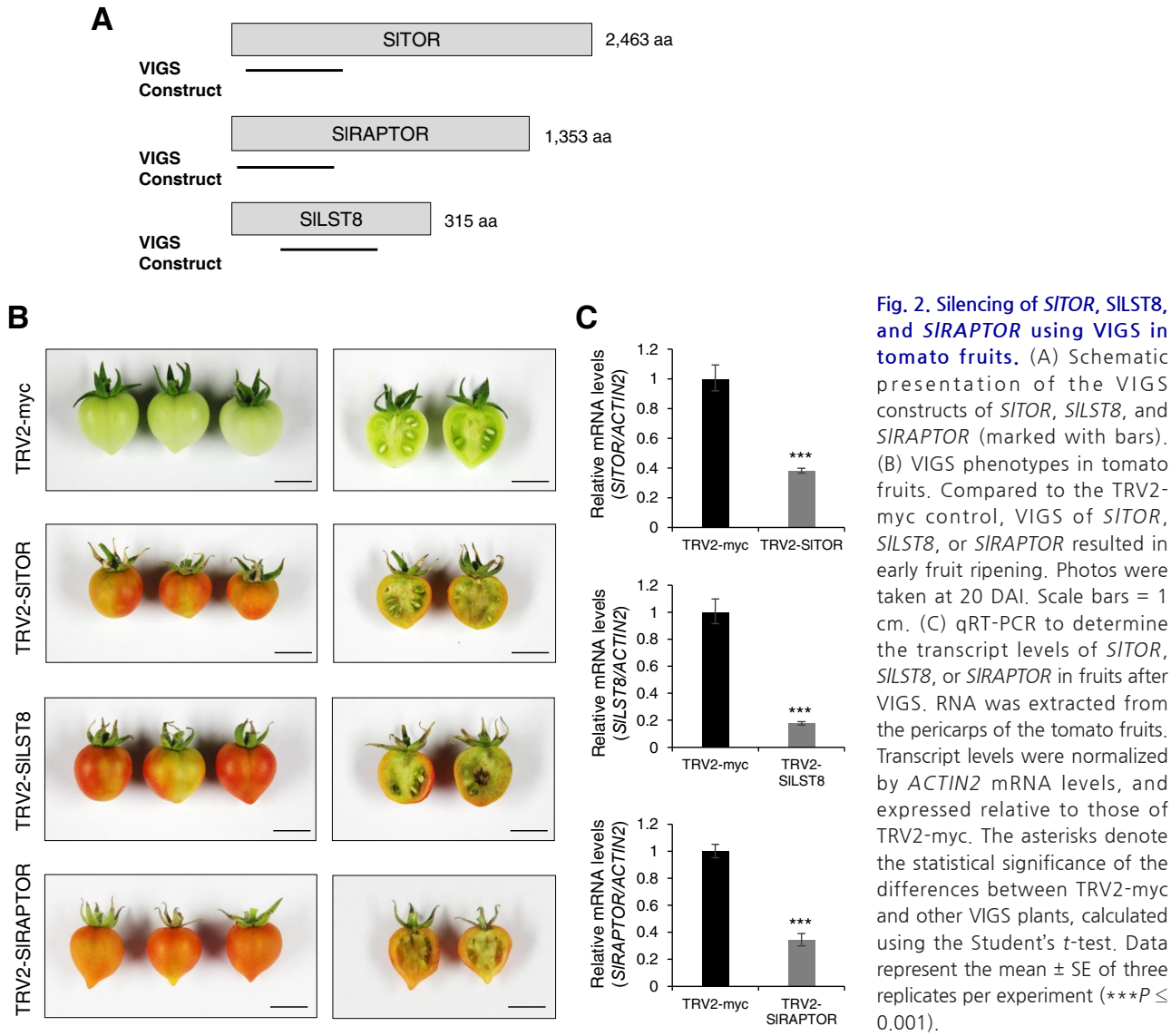

Fig. 2. Silencing of *SlTOR*, SlLST8, and *SlRAPTOR* using VIGS in tomato fruits. (A) Schematic presentation of the VIGS constructs of *SlTOR*, *SlLST8*, and *SlRAPTOR* (marked with bars). (B) VIGS phenotypes in tomato fruits. Compared to the TRV2-myc control, VIGS of *SlTOR*, *SlLST8*, or *SlRAPTOR* resulted in early fruit ripening. Photos were taken at 20 DAI. Scale bars = 1 cm. (C) qRT-PCR to determine the transcript levels of *SlTOR*, *SlLST8*, or *SlRAPTOR* in fruits after VIGS. RNA was extracted from the pericarps of the tomato fruits. Transcript levels were normalized by *ACTIN2* mRNA levels, and expressed relative to those of TRV2-myc. The asterisks denote the statistical significance of the differences between TRV2-myc and other VIGS plants, calculated using the Student's *t*-test. Data represent the mean ± SE of three replicates per experiment (***$P \leq 0.001$).

tissues of the fruits showed cell death symptoms in the TORC VIGS plants. Analysis of gene silencing by qRT-PCR showed that *SlTOR*, *SlLST8*, and *SlRAPTOR* mRNA levels were reduced to 38%, 17%, and 34% of the TRV2-myc level in TRV2-SlTOR, TRV2-SlLST8, and TRV2-SlRAPTOR fruits, respectively (Fig. 2C). VIGS of each TORC component gene did not affect transcript levels of the other members, suggesting that VIGS resulted in specific silencing of each gene and that depletion of one component did not influence the mRNA abundance of the other members (Supplementary Fig. S1). Collectively, these results suggest that TORC activity is important for the control of fruit growth and ripening.

Carotenoid accumulation in TORC-silenced fruits

Since *SlTOR*, *SlLST8*, and *SlRAPTOR*-silenced fruits showed early color change, compared to TRV2-myc control fruits, we observed accumulation of chlorophyll and carotenoid pigments in the fruits under confocal microscopy using specific excitation and emission wavelengths of each pigment (Fig. 3A). Protoplasts were generated from the pericarp of VIGS fruits at 20 DAI. Protoplasts from TRV2-SlTOR, TRV2-SlLST8, and TRV2-SlRAPTOR fruits showed strong green fluorescence from carotenoids but very weak red chlorophyll autofluorescence, whereas TRV2-myc protoplasts exhibited strong red autofluorescence from chloroplasts with almost no green fluorescence (Fig. 3A). Indeed, chlorophyll content was significantly reduced in TRV2-SlTOR, TRV2-SlLST8, and TRV2-SlRAPTOR fruits, compared to TRV2-myc control (Fig. 3B). Consistent with the visible fruit color change (Fig. 2C), these results suggest that early ripening occurred in TORC-silenced tomato fruits.

Next, we determined cellular levels of the main carotenoids in Micro-Tom fruits, such as lycopene, β-carotene, and lutein, in VIGS fruits (20 DAI) and red-ripened WT (wild type) Micro-Tom fruits (RED WT) using HPLC, followed by statistical analyses (Fig. 3C). The lutein levels were slightly lower in TRV2-SlLST8, TRV2-SlRAPTOR, and RED WT fruits than in TRV2-myc fruits. The β-carotene levels of RED WT and TORC

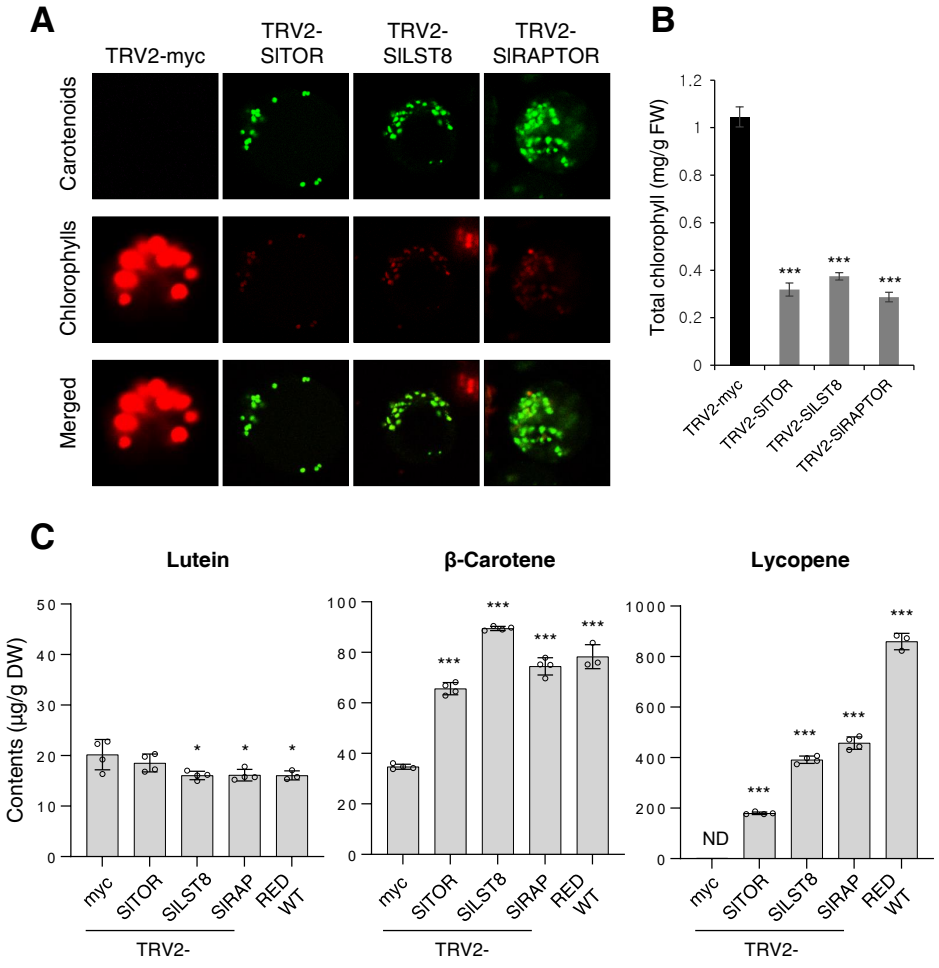

Fig. 3. Accumulation of carotenoids in tomato fruits after VIGS of TORC genes. (A) Autofluorescence of carotenoids (500-600 nm) and chlorophylls (650-750 nm) was observed in the pericarp protoplasts from VIGS fruits (20 DAI) using confocal microscopy. Green and red fluorescence indicate the carotenoids and chlorophylls, respectively. (B) Total chlorophyll content was measured using VIGS fruits (20 DAI). Data represent the mean ± SE of three replicates per experiment (***$P \leq 0.001$). FW, fresh weight. (C) Contents of diverse carotenoids were measured using HPLC in VIGS fruits (20 DAI) and red-ripened WT Micro-Tom fruits (RED WT). The β-carotene represents the sum of 13-cis-β-carotene, all-trans-β-carotene, and 9-cis-β-carotene. Data represent the mean ± SD of three to four replicates per experiment (*$P \leq 0.05$; ***$P \leq 0.001$; ND, non-detected). DW, dry weight.

VIGS fruits were relatively similar to each other, but significantly higher than those of TRV2-myc fruits. TORC VIGS fruits contained lower levels of lycopene compared with RED WT fruits, while TRV2-myc fruits (green color) have undetectable levels of lycopene. These results support the early ripening phenotype of TORC-silenced fruits. Considering the previous reports regarding carotenoid contents in Micro-Tom tomato (Leiva-Ampuero et al., 2020; Liang et al., 2020; Pinheiro et al., 2019), carotenoid accumulation in TORC VIGS fruits matched their early-ripening phenotypes and mimicked that of ripening WT fruits except its premature induction.

Upregulation of TFs, ethylene biosynthesis, and carotenoid biosynthesis genes

Ethylene biosynthesis and upregulation of TF genes are among the key events in the initiation of tomato fruit ripening. Since TORC silencing caused early onset of fruit ripening, we examined the expression patterns of TF genes and ethylene biosynthetic genes using qRT-PCR (Figs. 4A and 4B). After VIGS of *SlTOR*, *SlLST8*, and *SlRAPTOR*, the *RIN*, *NOR*, and *FUL1* mRNA levels in the fruits all increased at 14 DAI to reach the higher levels at 20 DAI (Fig. 4A). Expression of three ethylene biosynthesis genes, *ACS2*, *ACO1*, and *ACO3*, was highly upregulated at 14 DAI in TORC-silenced fruits, compared with TRV2-myc fruits, followed by a decrease at 20 DAI (Fig. 4B). Taken together, these results suggest that knock-down of TORC genes may cause early ripening phenotypes by stimulating the expression of the TF genes and ethylene biosynthesis genes.

Since mature tomato fruits primarily accumulate lycopene (Alba et al., 2005; Burns et al., 2003), and TORC silencing caused premature color change to orange/red (Fig. 1), we next examined the expression patterns of lycopene biosynthetic genes (Fig. 4C). qRT-PCR analyses were performed to determine the transcript levels of the following genes: phytoene synthase 1 (*PSY1*), *PSY2*, phytoene desaturase (*PDS*), ζ-carotene desaturase (*ZDS*), carotene isomerase (*CRTISO*), and ζ-carotene isomerase (*ZISO*). Expression levels of these genes were upregulated upon TORC silencing, and *PSY1* and *ZISO* transcript levels were particularly high in TORC-silenced fruits, compared with TRV2-myc control (Fig. 4C). During tomato fruit maturation, lycopene accumulates as the primary pigment, and the conversion of lycopene to other downstream products, α-carotene and β-carotene, is inhibited by reduced transcription of *SlLCYE* (involved in α-carotene synthesis) and *SlLCYB* (involved in β-carotene synthesis) (Stanley and Yuan, 2019). Based on qRT-PCR analyses using pericarps of tomato fruits at 23 DAI, the transcript levels of ly-

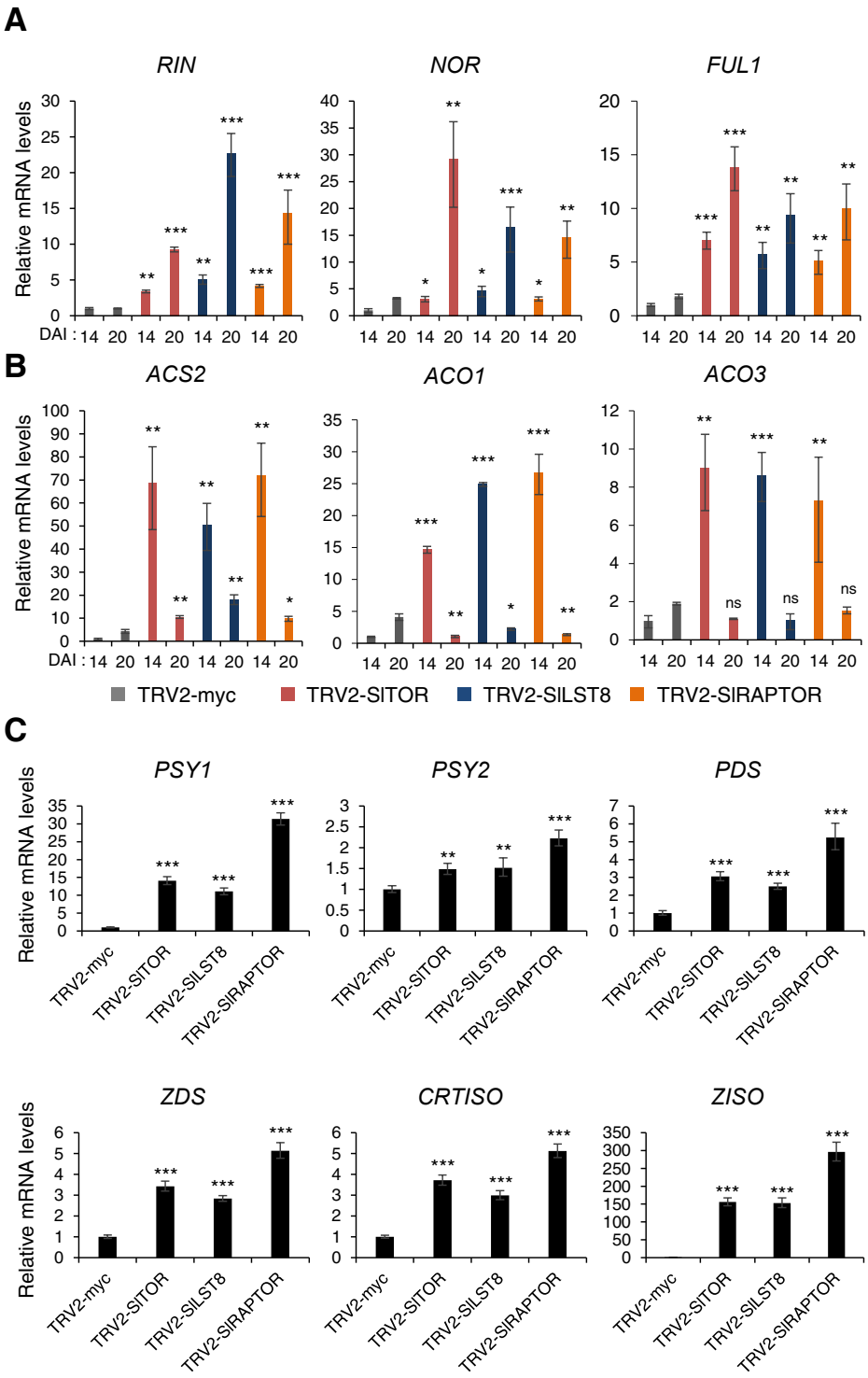

Fig. 4. Upregulation of ripening-related and carotenoid biosynthesis genes in fruits after VIGS of TORC genes. (A) qRT-PCR analyses of the transcripts levels of ripening-related transcriptional factor genes, ripening inhibitor (*RIN*), non-ripening (*NOR*), and fruitfull 1 (*FUL1*). RNA was isolated from the pericarps of TORC VIGS tomato fruits at ~14 and ~20 DAI. VIGS fruits reached the breaker stage and orange stage around 14 and 20 DAI, respectively. Transcript levels were normalized by *ACTIN2* mRNA levels and expressed relative to those of TRV2-myc. Data represent the mean ± SE of three replicates per experiment (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). (B) qRT-PCR analyses of the transcripts levels of ethylene biosynthesis genes, ACC synthase 2 (*ACS2*), ACC oxidase 1 (*ACO1*), and *ACO3*. RNA was isolated from the pericarps of TORC VIGS tomato fruits at ~14 and ~20 DAI. Data represent the mean ± SE of three replicates per experiment (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). (C) qRT-PCR analyses were performed to determine the transcripts levels of carotenoid biosynthesis genes: phytoene synthase 1 (*PSY1*), *PSY2*, phytoene desaturase (*PDS*), ζ-carotene desaturase (*ZDS*), carotene isomerase (*CRTISO*), and ζ-carotene isomerase (*ZISO*). RNA was isolated from the pericarps of the tomato fruits at 23 DAI. Data represent the mean ± SE of three replicates per experiment (**$P \leq 0.01$; ***$P \leq 0.001$).

copene ε-cyclase (*SlLCYE*) decreased, while those of lycopene β-cyclase 1 (*SlLCYB1*) and lycopene β-cyclase 2 (*SlLCYB2*) remained constant and slightly decreased, respectively (Supplementary Fig. S2). Collectively, these results suggest that silencing of TORC component genes accelerates fruit ripening by upregulating the transcription of ethylene biosynthesis genes, TF genes, and lycopene biosynthesis genes.

Alteration of cell wall metabolism in TORC-silenced fruits

Calcofluor white (CFW) is a fluorescent blue dye that stains cellulose in plant cell walls (Tang et al., 2015). To investigate cell wall softening in TORC-silenced fruits, we performed CFW staining to visualize cellulose in pericarp cell walls after VIGS (Fig. 5A). Outer pericarp tissues of TRV2-myc fruits showed strong fluorescence in the cell wall boundary after

CFW straining. However, TORC-silenced fruits showed a strong decrease in CFW fluorescence in pericarp tissues (Fig. 5A). Therefore, TORC silencing reduced cellulose deposition in pericarp cell walls, suggesting that cell wall softening occurs during the premature ripening process. Next, we performed qRT-PCR to examine the gene expression of cell wall modifying enzymes in VIGS fruits: β-mannosidase (*β-MAN*), β-galactosidase 4 (*β-GAL*), β-N-acetyl-D-hexosaminidase (*β-NHA*), expansin 1 (*EXP1*), polygalacturonase 2A (*PG-2A*), and cellulase 2 (*CEL2*) (Fig. 5B). These cell wall restructuring genes were highly expressed in ripened tomato (cv. Alisa Craig) (Kumar et al., 2018). All TORC-silenced fruits had dramatically increased transcript levels of these cell wall-modifying enzyme genes, indicating the progression of cell wall softening. Collectively, these results suggest that depletion of TORC components caused reduced cellulose deposition and up-regulation of cell wall softening genes, consistent with the early fruit ripening phenotypes.

Early ripening phenotypes observed in Moneymaker tomato fruits after VIGS of TORC genes

VIGS of TORC genes caused early ripening phenotypes in Micro-Tom tomato (Fig. 2), and we next examined whether the phenotype was reproduced in the commercial cultivar Moneymaker tomato. We performed VIGS in Moneymaker tomato fruits (stage 2) using the same VIGS constructs,

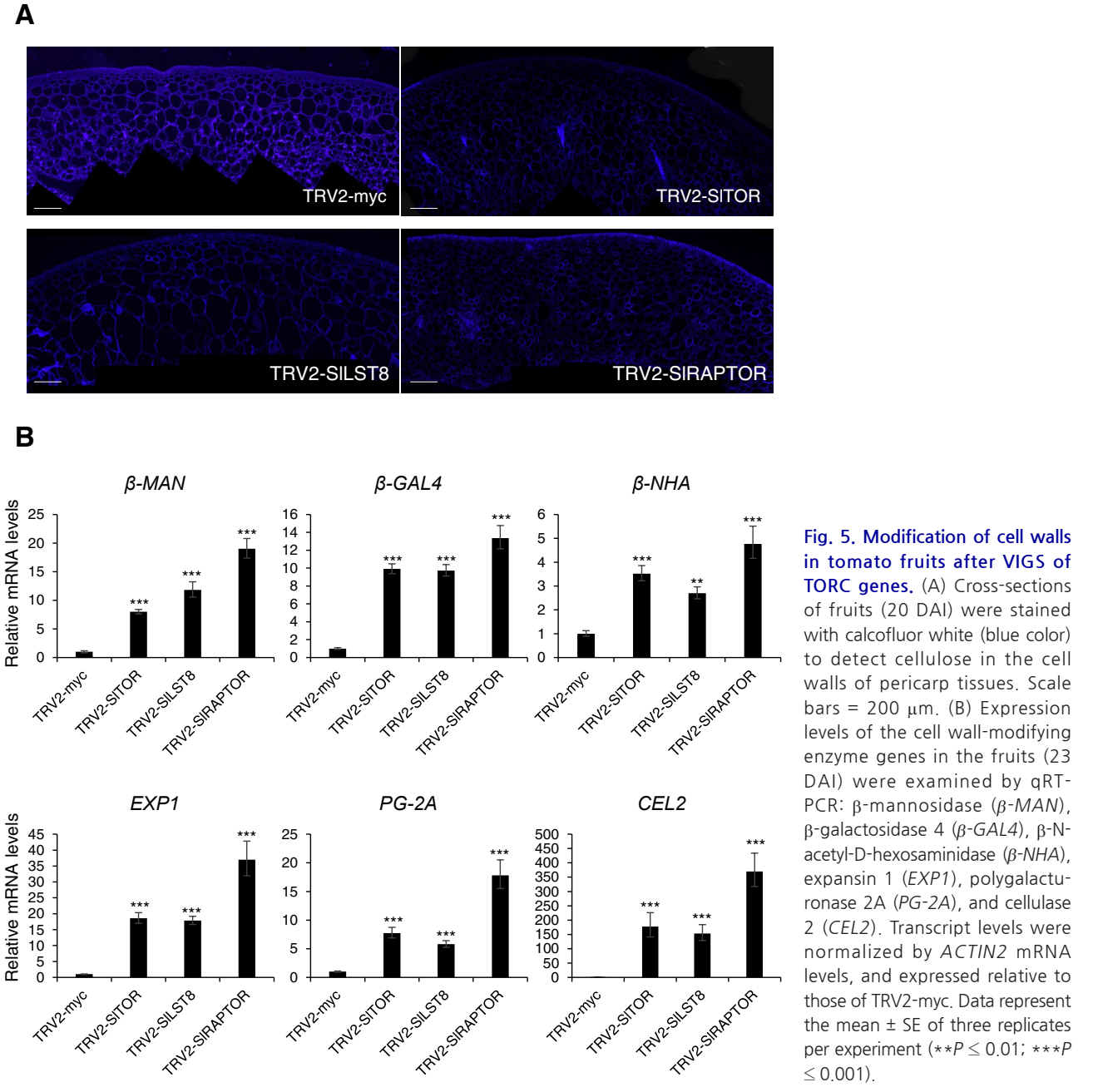

Fig. 5. Modification of cell walls in tomato fruits after VIGS of TORC genes. (A) Cross-sections of fruits (20 DAI) were stained with calcofluor white (blue color) to detect cellulose in the cell walls of pericarp tissues. Scale bars = 200 μm. (B) Expression levels of the cell wall-modifying enzyme genes in the fruits (23 DAI) were examined by qRT-PCR: β-mannosidase (*β-MAN*), β-galactosidase 4 (*β-GAL4*), β-N-acetyl-D-hexosaminidase (*β-NHA*), expansin 1 (*EXP1*), polygalacturonase 2A (*PG-2A*), and cellulase 2 (*CEL2*). Transcript levels were normalized by *ACTIN2* mRNA levels, and expressed relative to those of TRV2-myc. Data represent the mean ± SE of three replicates per experiment (**$P \leq 0.01$; ***$P \leq 0.001$).

TRV2-SlTOR, TRV2-SlLST8, and TRV2-SlRAPTOR. At 21 DAI, fruit color started to change in all TORC VIGS fruits without significant size differences from TRV2-myc fruits (Fig. 6A). At 25 DAI, TORC VIGS fruits showed red/orange coloration and reduced size, while TRV2-myc fruits were green, possibly reaching the mature green stage (Fig. 6B). Dissection of the fruits revealed cell death symptoms in the locular tissue, developing seeds, and placenta of TORC-silenced fruits. qRT-PCR demonstrated that transcript levels of *SlTOR*, *SlLST8*, and *SlRAPTOR* respectively decreased to 44%, 35%, and 23% of the TRV2-myc transcript levels after VIGS of each gene (Fig. 6C). Carotenoid accumulation in the pericarp was observed using confocal microscopy as described in Fig. 3A. Pericarp protoplasts isolated from the TORC-silenced Moneymaker fruits showed strong green fluorescence, suggesting carotenoid accumulation, while TRV2-myc protoplasts showed red chlorophyll autofluorescence (Fig. 6D). These results are consistent with the results obtained from Micro-Tom fruits (Fig. 3A). Thus, TORC silencing resulted in early ripening phenotypes in Moneymaker tomato, as shown in Micro-Tom

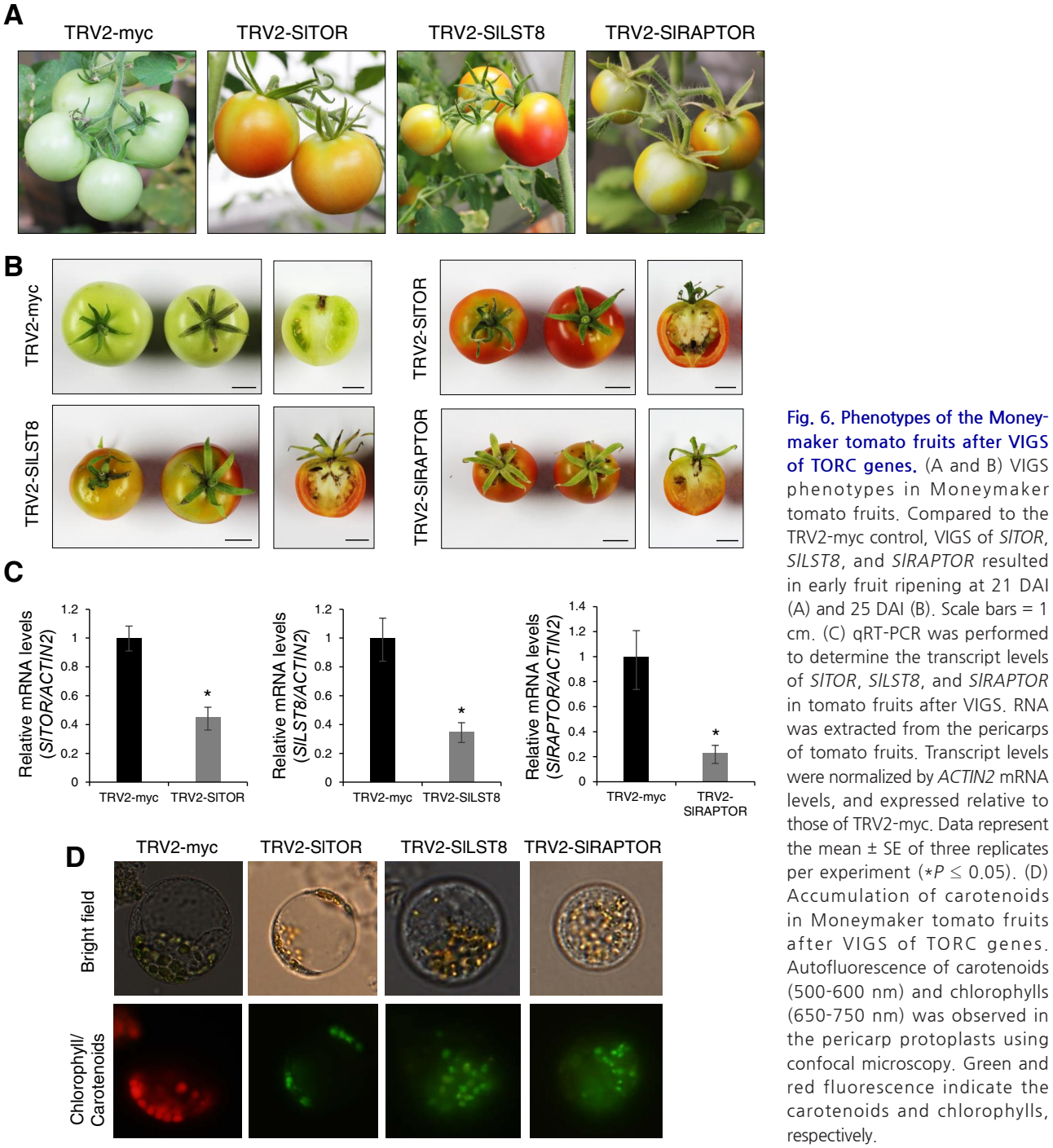

Fig. 6. Phenotypes of the Moneymaker tomato fruits after VIGS of TORC genes. (A and B) VIGS phenotypes in Moneymaker tomato fruits. Compared to the TRV2-myc control, VIGS of *SlTOR*, *SlLST8*, and *SlRAPTOR* resulted in early fruit ripening at 21 DAI (A) and 25 DAI (B). Scale bars = 1 cm. (C) qRT-PCR was performed to determine the transcript levels of *SlTOR*, *SlLST8*, and *SlRAPTOR* in tomato fruits after VIGS. RNA was extracted from the pericarps of tomato fruits. Transcript levels were normalized by *ACTIN2* mRNA levels, and expressed relative to those of TRV2-myc. Data represent the mean ± SE of three replicates per experiment ($*P \leq 0.05$). (D) Accumulation of carotenoids in Moneymaker tomato fruits after VIGS of TORC genes. Autofluorescence of carotenoids (500-600 nm) and chlorophylls (650-750 nm) was observed in the pericarp protoplasts using confocal microscopy. Green and red fluorescence indicate the carotenoids and chlorophylls, respectively.

tomato, confirming that TORC activity represses ripening in tomato fruits.

DISCUSSION

The TOR signaling pathway, which is conserved in all eukaryotes, coordinates cell growth and metabolism by integrating diverse signals regarding nutrients, energy, hormones, and stresses. Depletion of TOR activity caused growth arrest and premature senescence in Arabidopsis (Deprost et al., 2007; Fu et al., 2020; Menand et al., 2002). Similar growth inhibition occurred in tomato seedlings when treated with TOR inhibitors, such as KU63794, AZD8055, and Torin1 (Xiong et al., 2016). RNA sequencing analyses showed that DEGs (differentially expressed genes) included many genes related to cellular processes, such as photosynthesis, cell wall modification, and senescence, in tomato seedlings after TOR inhibitor treatment (Xiong et al., 2016). TOR regulation of these processes is conserved in many plant species, including Arabidopsis (Schepetilnikov and Ryabova, 2018). These results suggest that TOR kinase plays an important role in the vegetative growth of tomato plants. However, it was not clear whether the TOR pathway is involved in tomato fruit ripening. In this study, we discovered that the TOR complex critically modulates fruit ripening and aging in tomato.

Pericarps of stage 2 tomato fruits were infiltrated with *Agrobacterium* for VIGS of the TOR complex genes. Silencing of *SlTOR*, *SlLST8*, and *SlRAPTOR* all accelerated tomato fruit ripening, as shown by premature color change to orange/red (Fig. 2) and accumulation of the main carotenoid lycopene (Fig. 3C). Early carotenoid accumulation was accompanied by premature degradation of chlorophyll, suggesting cell wall softening in pericarp cells (Figs. 3 and 5). Gene expression analyses showed that TORC depletion highly upregulated the expression of ethylene biosynthesis genes, particularly *ACS2*, which encodes a rate-limiting enzyme ACC synthase. Transcript levels of *RIN*, *FUL1*, and *NOR* (well-known transcriptional factors that promote tomato fruit ripening), lycopene biosynthesis genes, and cell wall softening-related genes all increased upon TORC silencing. These results suggest that TORC-silenced tomato fruits undergo seemingly normal, but premature, ripening processes via transcriptional modulation of ripening-related genes. Previously, Xiong and Sheen (2013) reported that the glucose-TOR signaling pathway controls global transcriptional networks involved in primary and secondary metabolism, cell cycle, transcription, protein folding, and transport to activate meristems. Furthermore, it has been recently established that ethylene-insensitive protein 2 (EIN2), a direct target of TOR phosphorylation, functions as a central integrator of the glucose-TOR pathway for global transcriptional control of energy and biomass production (Fu et al., 2021). Collectively, the results of this study suggest that depletion of the TORC components accelerates tomato fruit ripening, possibly by orchestrating transcription of ripening-related genes, such as ethylene biosynthesis genes and TF genes.

NOR is an NAC TF that plays a critical role in tomato fruit ripening (Gao et al., 2020). Many NAC TFs have been shown to regulate leaf senescence in diverse plant species, including Arabidopsis, tomato, rice, and wheat (Garapati et al., 2015; Guo and Gan, 2006; Ma et al., 2018; Zhao et al., 2015; Zhou et al., 2013). Interestingly, the fruit ripening factor NOR was found to control leaf senescence in tomato (Ma et al., 2019). The *nor* mutation delayed dark-induced senescence, whereas *NOR* overexpression stimulated leaf senescence by modulating the expression of senescence-associated genes and chlorophyll degradation genes. These results suggest that fruit ripening/aging and leaf senescence may at least partly share TFs and gene regulatory networks (Gao et al., 2018; Ma et al., 2019).

Carotenoids are essential for light capture, photoprotection, and stabilization of the photosynthetic machinery in the photosynthetic tissues of plants (Esteban et al., 2015; Stanley and Yuan, 2019; Tanaka et al., 2008). Carotenoids also accumulate in flowers and fruits to attract animal interactors and are precursors for the synthesis of plant hormones, such as abscisic acid and strigolactones (Al-Babili and Bouwmeester, 2015; Lu and Li, 2008). Cellular levels of carotenoids are regulated by multiple mechanisms, but transcriptional control of carotenoid biosynthesis genes appears to be the main mechanism; approximately 40 putative regulators that affect the transcription of carotenoid biosynthesis genes have been identified (Stanley and Yuan, 2019). In this study, VIGS of TORC component genes, *SlTOR*, *SlLST8*, and *SlRAPTOR*, in tomato caused early fruit color change to orange/red (Fig. 2). Accordingly, expression levels of lycopene biosynthesis genes, *PSY1*, *PSY2*, *PDS*, *ZDS*, *CRTISO*, and *ZISO*, were all upregulated in the pericarp of Micro-Tom tomato (Fig. 4C).

Following the most recent reports regarding carotenoid contents in Micro-Tom tomato, which analyzed lycopene, β-carotene, and lutein as main carotenoids (Leiva-Ampuero et al., 2020; Liang et al., 2020; Pinheiro et al., 2019), we measured the contents of the three types of carotenoids using HPLC (Fig. 3C). TORC-silenced fruits contained similar levels of β-carotene and lutein, but lower levels of lycopene, compared with the red-ripened WT Micro-Tom fruits. The control TRV2:myc fruits accumulated undetectable amounts of lycopene in the pericarp, consistent with their green color, but they accumulated low levels of lutein and β-carotene. The differences between TRV2:myc and TORC VIGS fruits were similar to the previous reports between young and ripened Micro-Tom fruits (Leiva-Ampuero et al., 2020; Liang et al., 2020), suggesting that early ripening of TORC VIGS fruits is accompanied by normal patterns of carotenoid accumulation. TORC VIGS fruits never reached the red-ripened stage of WT Micro-Tom fruits, which explains lower lycopene accumulation (Fig. 3C). Collectively, these results support the early ripening phenotypes caused by TORC silencing.

Early ripening phenotypes after TORC silencing were observed in both Micro-Tom and the commercial cultivar Moneymaker tomato varieties (Figs. 2 and 6). Micro-Tom has been used as a model system for tomato research because of its small size, short life cycle, and efficient genetic transformation (Shikata and Ezura, 2016). Micro-Tom tomato, displaying a dwarf phenotype, is known to have mutations in the *SELF-PRUNING* and *DWARF* genes (Lima et al., 2004; Pnueli et al., 2001). *SELF-PRUNING* encodes a 23 kDa CETS (CENTRORADIALIS/TERMINAL FLOWER 1/SELF-PRUNING) family

modulator that determines the potential of the shoot apical meristem for continuous growth (Pnueli et al., 2001). The *DWARF* gene encodes a P450 protein that catalyzes the C-6 oxidation of 6-deoxocastasterone to castasterone in brassinosteroid biosynthesis (Bishop et al., 1999). Furthermore, Micro-Tom tomato has an additional mutation that decreases the internode length without affecting the active gibberellin levels (Martí et al., 2006). Irrespective of these mutations in Micro-Tom tomato, VIGS of TORC genes caused similar phenotypes in fruits of both Micro-Tom and Moneymaker tomato varieties (Figs. 2, 4, and 6). Therefore, TORC silencing phenotypes were conserved in Micro-Tom tomato despite the presence of several mutations in its genome, indicating the importance of TORC function in plant development.

We observed in this study that TORC deficiency accelerates tomato fruit ripening, accompanied by transcriptional induction of a plethora of genes involved in fruit ripening. TOR regulates ethylene signaling via EIN2 receptor in Arabidopsis (Fu et al., 2021). Inactivation of TOR by AZD8055 causes accumulation of ACS2 and ACS6 that are critical enzymes for ethylene biosynthesis (Zhuo et al., 2020). These results suggest that TOR modulates fruit ripening through ethylene biosynthesis and signaling. A recent study demonstrated that overexpression of PpSnRK1α promotes tomato fruit ripening, revealing a positive upstream regulator (Yu et al., 2018). The authors showed that direct interaction between PpSnRK1α and RIN TF is important for accelerated fruit ripening. It has been known that SnRK1 and TORC function antagonistically; knock-down of TORC increases SnRK1 activity (Robaglia et al., 2012). Thus, tomato fruit ripening may be controlled by TORC through the ethylene pathway, but also by SnRK1 directly through RIN TF. Furthermore, López-Vidal et al. (2020) found the relationship between fruit ripening and autophagy in sweet California pepper (*Capsicum annum*). Since inactivation of TOR promotes autophagy, the finding suggests another mechanism of TOR-regulation on fruit ripening. Further study would illustrate how tomato fruit ripening is controlled by upstream regulators including TORC.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

This research was supported by the Basic Science Research Program (2018R1A6A1A03025607) and the Mid-Career Researcher Program (2022R1A2C1009088) from the National Research Foundation (NRF) of the Republic of Korea.

AUTHOR CONTRIBUTIONS

I.C., C.S.A., and D.H.L. performed the molecular and biochemical analyses. H.S.L. gave feedbacks on experimental data and writings. S.A.B., J.W.J., and J.K.K. performed the HPLC analyses of carotenoid contents. I.C. and H.S.P. wrote the manuscript.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

ORCID

Ilyeong Choi https://orcid.org/0000-0002-9243-6279
Chang Sook Ahn https://orcid.org/0000-0002-4829-3485
Du-Hwa Lee https://orcid.org/0000-0003-4106-7730
Seung-A Baek https://orcid.org/0000-0003-4045-6048
Jung Won Jung https://orcid.org/0000-0003-2620-6480
Jae Kwang Kim https://orcid.org/0000-0003-2692-5370
Ho-Seok Lee https://orcid.org/0000-0002-9620-1522
Hyun-Sook Pai https://orcid.org/0000-0003-1345-5840

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