Ethylene Response Factor SlERF.D6 promotes ripening initiation and ethylene response through downstream transcription factors SlDEAR2 and SlTCP12

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Abstract

Ripening is crucial for the development of fleshy fruits that release their seeds following consumption by frugivores and are important contributors to human health and nutritional security. Many genetic ripening regulators have been identified, especially in the model system tomato, yet more remain to be discovered and integrated into comprehensive regulatory models. Most tomato ripening genes have been studied in pericarp tissue, though recent evidence indicates that locule tissue is a site of early ripening-gene activities. Here we identified and functionally characterized an Ethylene Response Factor gene, *SlERF.D6*, by investigating tomato transcriptome data throughout plant development, emphasizing genes elevated in the locule during fruit development and ripening. *SlERF.D6*loss-of-function mutants resulting from CRISPR/Cas9 gene editing delayed ripening initiation and carotenoid accumulation in both pericarp and locule tissues. Transcriptome analysis of lines altered in *SlERF.D6* expression revealed multiple classes of altered genes including ripening regulators, in addition to carotenoid, cell wall and ethylene pathway genes, suggesting comprehensive ripening control. Distinct regulatory patterns in pericarp versus locule tissues were observed indicating tissue-specific activity of this transcription factor. Analysis of SlERF.D6 interaction with target promoters revealed an AP2/ERF transcription factor (*SlDEAR2*) as a target of SlERF.D6. Furthermore, we show that a third transcription factor gene, *SlTCP12*, is a target of SlDEAR2, presenting a tri-component module of ripening control.

Significance

A tomato ETHYLENE RESPONSE FACTOR gene, *SlERF.D6*, encodes a positive and primary regulator of tomato fruit ripening influencing all measured ripening changes and numerous corresponding known genes contributing to ripening phenotypes via a cascade of multiple transcription factors. The resulting SlERF.D6 ripening regulatory module was further placed in context of known ripening regulators including ethylene and previously described essential ripening transcription factors.

Introduction

Ripening in fleshy fruits is a complex and tightly controlled genetic program involving a range of changes in chemistry and physiology often typified by accumulation of nutritional compounds, synthesis and accrual of pigments, reduction in photosynthesis and photosynthetic pigments, texture modifications, and alterations in volatile aromatic profiles (1, 2). Traditionally, fleshy fruits are classified as climacteric or non-climacteric based on the presence or absence of elevated respiration and ethylene levels, respectively, at the onset of ripening (3, 4). Climacteric fruits such as tomato, apple, banana, and avocado display a rapid rise in respiration and concomitant increase in ethylene production upon initiation of ripening, whereas non-climacteric fruits including citrus, strawberry, and grape do not, although they still may respond to exogenous ethylene (5). Much understanding of the ripening regulatory mechanism underlying climacteric fruit maturation has been developed using the crop model tomato (*Solanum lycopersicum*) (2, 6).

Many transcription factors (TFs; Dataset S1) have been associated with ripening regulation. These TFs influence all or subsets of fruit maturation and ripening characteristics to varying degrees when altered via natural mutation, targeted repression or gene-editing. Yet, while many components are in hand, a clear regulatory network remains to be identified. In addition to TFs, epigenomic changes (7) and the plant hormone ethylene are necessary for climacteric fruit ripening. Silencing of ethylene biosynthesis genes *ACS2* or *ACO1* results in unripe fruits also impairing the expression of additional ethylene-regulated biosynthesis genes including *ACS4* and *ACO3* (8–11). The *Never-ripe* (*Nr*) tomato fruit ripening mutant gene encodes an ethylene receptor (LeETR3) crucial in ethylene dependent ripening and contributes to ripening in conjunction with another predominantly fruit expressed ethylene receptor, LeETR4 (12, 13). Other ethylene regulatory genes are known to repress ethylene signaling and ripening. For example, repression of the tomato *AP2a* gene causes accelerated ripening mediated by interaction with miRNA172 (14, 15). Silencing *SlEBF1* or *SlEBF2* expression also causes earlier fruit ripening whereas over-expression of *SlEBF3* impedes fruit ripening by reducing the abundance of EIL proteins (16, 17). It has recently been shown that CRISPR edited *SlEIN2* mutants displayed complete cessation of ripening, which was partially rescued by *slebf1* but not *slebf2* or *slebf3* (18). This same study demonstrated a molecular connection between ethylene and the *RIN*, *NOR* and *FUL1* transcription factors mediated by *EIL* ethylene signaling transcription factors.

Ethylene signal transduction additionally includes transcriptional regulators known as Ethylene Response Factors (ERFs) (19, 20) acting downstream in the signaling cascade and are more immediate regulatory mediators of ethylene response. ERFs belong to the large AP2/ERF multi-gene family characterized by the presence of the AP2/ERF domain consisting of 40–70 conserved amino acids involved in DNA binding (21, 22). In tomato, LeERF1, SlERF6, SlERF.B3 and SlPit4 have been shown to play important roles in hormone signaling and fruit ripening (23–26), while SlERF.F12 was recently shown to negatively influence ripening via histone modification at ripening gene loci (27) in addition to SlERF.D7 that positively influences ripening by activating *SlARF2A/B* (28). With at least 55 fruit ripening-related ERFs in the tomato genome (29, 30), the understanding of ERF family genes in fruit development and ripening is far from complete as is their interaction with other regulators in the larger ripening regulatory cascade.

It is notable that most molecular ripening studies focus on the fruit flesh which is the carpel in tomato or expanded floral extra-carpelary tissues comprising the flesh of, for example, apple and strawberry (31). Only recently have investigations focused more holistically on additional fruit tissues including placenta, locule and inner carpel tissues (32–34). Though the transition from solid to viscous locule tissue has long been associated with early fruit maturation in advance of visible color change (35), locule tissue has been often overlooked for molecular analyses because of practical difficulties stemming from the nature of the viscous tissue and embedded seed (2). Nevertheless, transcriptome profiling of locule tissue throughout development has revealed that key ripening regulators including *RIN*, *NOR*, and *DML2* are induced at the mature green (MG) stage, several days prior to the onset of color and ethylene induction (32), suggesting that locule tissue is a site of early ripening-gene activities.

Here we report the functional characterization of an ERF family gene, *SlERF.D6*, which is strongly induced in pericarp in concert with ripening but is first induced in locule prior to the MG stage, more than a week before changes in ripening physiology occur in the fruit and consistent with the timing of ripening-related demethylation and competence to ripen early in response to exogenous ethylene. We used CRISPR/Cas9 gene-editing to generate loss-of-function mutants of *SlERF.D6* and performed fruit transcriptional profiling and DNA affinity purification sequencing to clarify the role of *SlERF.D6* in tomato fruit ripening. Our results suggest that *SlERF.D6* is a positive regulator of fruit ripening and plays important roles in both pericarp and locule tissues in part through regulation of at least two additional transcription factors that operate with influence from additional ripening transcription factors and ethylene.

Results

SlERF.D6 positively influences fruit ripening

Much molecular and physiological ripening research has focused largely or exclusively on the pericarp but recent analyses indicate locule tissue undergoes similar molecular changes prior to pericarp maturation (2, 32, 33, 36, 37). As examples, *RIN-MADS*, *NOR-NAC, GRAS38* and *SlLOB1* all influence ripening traits and all display their first elevated expression in tomato locule tissue preceding their induction in pericarp (Fig. S1a-d), suggesting that ripening regulators often first respond in locule tissues.

We searched the Tomato Expression Atlas (https://tea.solgenomics.net/) (32) for uncharacterized TFs with similar expression patterns to early locule expression ripening TFs and identified 12 for which we attempted to generate CRISPR/Cas9 edited mutations in tomato cultivar *Ailsa Craig* (Dataset S2). The edited mutants showed diverse phenotypes: CRISPR editing of *SlWOX14* (encoding a WUSCHEL-related homeobox protein), *SlHsfA6b* (encoding a heat shock factor), and *SlERF.D6* (encoding an ethylene response factor) delayed fruit ripening (Fig. 1; Fig. S1e); editing of *SlZHD23* (encoding a zinc finger family protein) delayed plant development and altered morphology (Fig. S1f); editing of *SlARR11* (encoding a two-component response regulator) displayed reduced pigments in flowers and fruits (Fig. S2), confirming prior transgenic over-expression results (38).

We focused on *SlERF.D6* (*Solyc04g071770*), given the stronger ripening delay in edited lines. *SlERF.D6* was additionally repressed in both the ripening deficient *rin* and *nor* mutants (Fig. S3a) and was upregulated by exogenous ethylene supplied to MG fruit and downregulated by ethylene inhibitor 1-MCP, indicating that this gene is positively ethylene responsive (Fig. S3b). Examination of reported ChIP-Seq data (2, 39) indicates that the upstream sequences of *SlERF.D6* bind TFs RIN-MADS, NOR-NAC and TAGL1 (Fig. S3c). It is noteworthy that all three TFs result in ripening inhibition when mutated (2), and RIN-MADS and TAGL1 have been shown to physically interact (40). In addition to the AP2/ERF DNA binding domain, analysis of SlERF.D6 protein structure using MobiDB (41) and PLAAC (42) revealed four intrinsically disordered regions (IDRs), two of which were predicted to have Prion-like domains (PrLD) (Fig. S4). Proteins containing nucleic acid binding motifs, IDRs, and PrLDs are required for the formation of biomolecular condensates through liquid-liquid phase separation (43, 44). It is noteworthy that

condensate formation has been previously associated with the regulation of auxin (44) and ethylene signaling and response in Arabidopsis (45). Together these observations led us to hypothesize that SlERF.D6 is an important contributor to tomato fruit maturation and ripening control in part through interaction with previously described central ripening TFs.

Delayed fruit ripening in SlERF.D6 gene-edited mutants

To assess *SlERF.D6* function, three independent CRISPR edited (Cr) lines (*Cr3*, *Cr8, Cr7*) harboring distinct mutations in the gene were selected for further characterization (Fig. 2a). All three lines showed similar delayed fruit ripening initiation of approximately 5 days, uneven ripening and slower attainment of full color (Fig. 2b, 2c). We selected the *Cr3* and *Cr8* lines for detailed analysis. Consistent with delayed pigment accumulation, peak ethylene evolution in the mutants was reduced by 70% as compared to WT controls, occurring 3 days later than WT (Fig. 2d). Fruit texture analysis showed softening of fruits was delayed significantly during early ripening but achieved similar firmness to WT controls in very mature fruit at 50 days post anthesis (DPA) (Fig. 2e). Though substantially delayed in ripening onset and color development, the mutant and WT fruit shared similar post-harvest water loss phenotypes and cuticle integrity as assessed by Toluidine blue staining (Fig. S5).

We further examined carotenoid accumulation in *SlERF.D6* mutant lines. Pericarp tissues of all *Cr* mutants initially accumulated less lycopene (red) and β-carotene (orange) when compared to WT fruits (Fig. 2f, 2g), though by 50 DPA carotenoid levels in very mature mutant and WT fruit became similar. In addition to changes in pericarp coloration and carotenoid profiles, *SlERF.D6* CRISPR mutants also displayed altered locule pigmentation (Fig. S6). Mutant lines presented lighter green locules than WT at both 35 DPA and 39 DPA (Fig. S6a) resulting from reduced chlorophyll a/b contents (Fig. S6b, c). The predominant carotenoids in the locules, lycopene, beta-carotene and lutein, varied with lutein notably lower in early ripening mutant fruit with changes largely overcome by late ripening (50 DPA) (Fig. S6d-g).

Transcriptome profiling of SlERF.D6 mutants

To identify transcriptome changes resulting from *SlERF.D6* mutation, RNA-Seq analysis was performed on fruit pericarp and locular tissues from WT and two independent mutant lines at the 29 DPA, 33 DPA, 35 DPA, 39 DPA, and 42 DPA stages (Fig. S7a-b). In WT fruit 33 DPA and 35 DPA correspond to the Mature Green (MG) and Breaker (BR) stages, respectively. To align fruit developmentally for comparison of similar appearing fruit of different ages, additional RNA-Seq was conducted on 46 DPA (~ BR + 7) and 54 DPA (~ BR + 15) mutant and 50 DPA (~ BR + 15) WT (Fig. S7a-b) fruit. Principal component analysis (PCA) showed that 29 DPA and 33 DPA belonged to the pre-ripening cluster in pericarp tissue among all lines. PCA divergence began at 35 DPA when WT initiated ripening (BR) while mutants remained more similar to the unripe MG stage (Fig. S7a). In locule tissue, the divergence began at 33 DPA (Fig. S7b) as some ripening factors altered expression (Fig S1a-d). All genotypes clustered at the very end of measured development, consistent with their similar appearance and coloration (Fig. 2b).

Analysis of differentially expressed genes (DEGs; fold change ≥ 2 and adjusted $P < 0.05$) in all tissues revealed a substantial perturbation of the transcriptome by the *SlERF.D6* mutation (Dataset S3). We focused on differences in 35 DPA and 33 DPA pericarp and locule tissues, respectively, as this is when ripening changes became manifest in WT fruit but were still not evident in the mutants. A total of 874 upregulated and 683 downregulated genes were identified in 35 DPA pericarp tissues, as well as 714 and 1114, respectively, in 33 DPA locular tissues, in both *Cr3* and *Cr8* lines compared to the WT controls (Figure S6c-d).

Gene Ontology (GO) term enrichment analysis was conducted for the identified DEGs in 35 DPA pericarp and 33 DPA locule. A total of 19 and 59 GO terms enriched in upregulated and downregulated genes, respectively, were identified in 35 DPA pericarp tissues, as well as 26 and 41, respectively, in 33 DPA locular tissues (Dataset S4). Cell wall related and membrane integrity related GO terms were enriched in upregulated genes in both tissues (Fig. S7e-f). Pigment biosynthetic process, fruit ripening, carotenoid metabolism, flavonoid biosynthetic and ethylene biosynthetic processes were enriched in downregulated genes in 35 DPA mutant pericarp tissue as compared to WT (Fig. S7g). For 33 DPA locule tissue, photosystem associated pathways were the predominant GO terms enriched for downregulated genes (Fig. S7h). The photosystem and carotenoid gene expression changes were consistent with the coloration changes observed in the locule tissue (Fig. S6). Ethylene activated signaling pathway was also downregulated in this tissue (Fig. S7h).

Altered cell wall and ripening-associated genes in SlERF.D6 mutant fruit

Upon further mining of our GO analysis results, we found that despite delayed ripening initiation, the mutants displayed some upregulation of cell wall genes prior to ripening. Investigation of cell wall associated DEGs revealed six and two of the most differently expressed genes in the pericarp and locule tissue of the mutants versus WT, respectively (Fig. S8a, b). These genes encode cellulose synthases (Fig. S8a, h), COBRA-like protein (Fig. S8d), expansin-associated proteins (Fig. S8e, i), BURP domain-containing protein (Fig. S8f), pectin acetylesterase like protein (Fig. S8g), and endotransglucosylase/XTH8 (Fig. S8j). All have reported or suggested roles in cell wall structure and/or remodeling (46–50), were upregulated at earlier ripening stages compared to WT and achieved WT expression levels during later development and ripening in the mutants. These changes alone obviously were not sufficient to alter fruit texture and some, for example increased expression of cellulose synthase, would be consistent with enhanced cell wall integrity more typical of pre-ripening fruit.

Investigation of additional cell wall regulators which were reported recently to be important for textural changes of ripening tomatoes (36), showed that *LOB1, EXP1, E6, CEL2, PL1-27*, and *GASA* were all suppressed in the mutants at early stages of ripening in pericarp (Fig. S9a-c). In locule, *PL1-27* and *GASA* were notable for being suppressed even earlier from 29 DPA to 35 DPA, consistent with the earlier expression of *SlERF.D6* in this tissue, with continued suppression into later development (Fig. S9d, e).

PG2a and *PL*, classified under the GO term “fruit ripening”, were also inhibited in early ripening (Fig. S9f, g). Another four ripening- and ethylene-associated genes, *ACS2, ACS4, E8* and *E8-Homolog* were

repressed at the same stages (Fig. S9h-k). The decreased expression levels of *ACS2* and *ACS4* likely contributed to the reduced production of ethylene observed in the mutants. Additionally, *RIN-MADS*, *NOR-NAC*, *CNR-SPL*, *DML2 and FUL1-MADS* were notable for being strongly suppressed in 35 DPA pericarp and 33 DPA locule tissues (Fig. S9l, m), consistent with the ripening delay observed in mutant fruit.

Altered ethylene signaling and photosystem associated pathways in the locular tissues of the SlERF.D6 mutants

Based on GO term analysis of downregulated genes in 33 DPA locule (Fig. S7h), sixty DEGs in the ethylene-activated signaling and photosystem associated pathways (Fig. S10a, b) were identified and those most significantly altered were shown (Fig. S10c-e). Ethylene receptor *ETR4* was consistently repressed from 33 DPA to 39 DPA (Fig. S10c), while receptor *ETR3* was most highly suppressed at 33 DPA (Fig. S10d). Both receptor genes are involved in ripening (51–53). At 33 DPA, *EIL4* and *ERF.E1*, primary and secondary ethylene responsive factors, along with one AP2/ERF transcription factor *SlDEAR2* and phosphate transporter were also inhibited (Fig. S10d).

Genes encoding photosystem proteins including chlorophyll a-b binding proteins (CABs), PsaD subunit of photosystem I, photosystem II 10kDa polypeptide (PsbR) were suppressed at early ripening, consistent with the lower chlorophyll content in locule tissues of the mutants (Fig. S10e; Fig.S6a, b). Two other photosystem associated genes, *ERD15* (encoding an early response to dehydration 15) and *HSMT* (encoding a homocysteine S-methyltransferase) were also repressed in early ripening (Fig. S10e).

Hindered pigment biosynthetic and metabolic processes in SlERF.D6 mutants

Consistent with the clear carotenoid phenotypes observed in the mutants, twenty DEGs related to pigment biosynthetic, carotenoid metabolic and flavonoid biosynthetic processes were identified in 35 DPA pericarp (Fig. S11a, b). Five genes, *1-D-deoxyxylulose 5-phosphate synthase (DXS)*, *pheophytinase (PPH)*, *phytoene synthase 1 (PSY1)*, *alcohol acyl transferase (AAT1)*, and *green flesh (GF)* were among the strongest differentially expressed (Fig. S11b-f). *GF* encodes a stay-green protein associated with chlorophyll retention (54) and whose repression is consistent with the delayed color change (Fig. 2) of *SlERF.D6* mutant fruit.

Given the carotenoid phenotypes of fruit locules, we analyzed the expression of carotenoid biosynthetic genes in that tissue as well (Fig. S12). Several carotenoid pathway genes were altered in expression in *SlERF.D6* mutant lines from prior to committed carotenoid synthesis (*DXS*) to key rate limiting steps (*PSY1*, *ZISO*) through downstream pathway activities (*CrtR-B2*) (Fig. S12a-d), all consistent with the altered coloration phenotypes of delayed and modified carotenoid accumulation in the pericarp and locule, respectively.

Accelerated ripening initiation and reduced fruit size in SlERF.D6 overexpression lines

To confirm the positive role of *SlERF.D6* in ripening, we generated transgenic *SlERF.D6* overexpression (OE) plants and three independent lines were selected for further analysis (Fig. 3a). The OE lines

displayed accelerated ripening initiation (Fig. 3b, c) along with earlier ethylene induction and enhanced fruit softening (Fig. 3d, e). OE lines also presented smaller fruit size and reduced fruit weight (Fig. 3f, g). OE lines additionally showed altered plant vegetative phenotypes including epinasty (Fig. S13a), constitutive triple response seedlings suggestive of elevated ethylene and/or response (Fig. S13b), elongated and curled leaf morphology (Fig. S13c), and larger flower size with longer sepals (Fig. S13d, e). All fruit phenotypes were opposite of those observed in gene-edited mutant lines and vegetative phenotypes were consistent with elevated ethylene response phenotypes.

Fruit RNA-Seq data showed numerous ripening associated genes were upregulated in a representative overexpression line, *OE1* (Dataset S5, Fig. S14). Ripening regulatory transcription factors *RIN-MADS*, *NOR-NAC*, *CNR-SPL* and *DML2* were upregulated at one or more stages of fruit ripening (Fig. S14a-d) reiterating the positioning of SlERF.D6 in context of previously described key regulators and consistent with the gene-editing loss-of-function results. Carotenoid biosynthesis pathway gene *PSY1* was also upregulated in *OE1* at 33 DPA and 35 DPA (Fig. S14e). Meanwhile, *LCYB1* was downregulated in the same tissues (Fig. S14f). As *PSY1* and *LCYB1* are regulated positively and negatively by ethylene, respectively (Alba et al., 2005), to promote carotenoid synthesis and block lycopene catabolism, these observations are consistent with a role of *SlERF.D6* in mediating ethylene regulation which in turn controls fruit lycopene accumulation. Fruit cell wall metabolism genes *PG2a* and *PL* were also upregulated at the beginning of maturation in *OE1*, consistent with enhanced and early softening (Fig. 3e; S14g, h). Ethylene biosynthesis and responsive genes *ACS2, ACS4, E4*, and *E8* were upregulated early in *OE1* fruit as well (Fig. S14i-l).

Primary target genes of SlERF.D6

DNA affinity purification sequencing (DAP-Seq) was carried out to further investigate the potential targets of SlERF.D6. A total of 11,772 highly reliable binding sites associated with 3,317 target genes in the tomato genome were identified (Supplementary Data 5). The most significant motif (E-value = 8.7×10^{-1037}) found by the programs MEME, DREME, and CentriMo (55) in the DAP-Seq data was the GCC box (Fig. S15a), which is known as the conserved binding site of ERFs (56, 57).

Among the targets identified by DAP-Seq, multiple contribute to or are associated with fruit ripening (Dataset S6): AP2/ERF family members (*ERF.F7*, *Solyc03g006320; SlERF3-19*, *Solyc03g119800; SlDEAR2*, *Solyc04g078640; ERF.H9*, *Solyc07g042230; SlERF12-4*, *Solyc12g009490*), tetratricopeptide repeat protein (*TPR5, Solyc05g008420*), ABA receptors (*SlRCAR12*, *Solyc03g007310; SlRCAR15*, *Solyc05g052420*), auxin associated genes (*ARF5*, *Solyc04g081240*; *ARF7*, *Solyc07g042260*; *SAUR65*, *Solyc11g011730*), bHLH transcription factors (*bHLH3*, *Solyc01g081090*; *bHLH31*, *Solyc04g007430*; *bHLH61*, *Solyc09g089870*), zinc finger transcription factors (*ZFP45*, *Solyc06g069440; ZFP69*, *Solyc11g069340*), aldehyde dehydrogenase (*SlALDH2B1*, *Solyc02g086970*), and UDP-glycosyltransferase (*SlUDPGT19*, *Solyc03g078780*).

Comparing with the transcriptome data of loss-of-function mutants, we identified 428 DEGs that had SlERF.D6 consensus binding sites on their promoters while 1197 DEGs that had binding sites in their gene bodies (Fig. S15b, c; Dataset S7). By investigating genes with binding sites in their bodies, we found twelve previously and mostly functionally described genes: *SlTCP12, SlARF2a, SlGGPPS2, SlZDS, CrtR-B1, SlPL, Expansin1, SlE4, SlE8, SlERF.A2, SlERF.A3*, and *ERF.F12* (Fig. S16). Gene sequences themselves are known to serve as sites of regulatory information as has been widely reported in human cells, maize, rice, wheat, and Arabidopsis (58–63).

Among genes with promoter binding sites included three target genes of SlERF.D6, *SlDEAR2, SlRCAR12*, and *SlUDPGT19* (Fig. S15d-f) that were significantly downregulated in edited lines (Fig. S15g-i). UDP-glycosyltransferase has been reported to play a role in fruit ripening (64, 65). Co-silencing *SlRCARs* including *SlRCAR12* delayed fruit ripening in tomato (66). AP2/ERF transcription factor *SlDEAR2* was previously linked with fruit ripening (67) as a possible regulator of the fruit ripening associated TF *SlTCP12 (Solyc11g020670)* based on co-expression and yeast one hybrid analyses (68).

Impaired fruit ripening in CRISPR edited mutants of SlERDF.D6 target genes

Because the TF *SlDEAR2* is a target of SlERF.D6 and has been implicated in ripening but not functionally studied to date, we generated CRISPR/Cas9 editing mutations at the *SlDEAR2* locus in tomato cultivar Ailsa Craig. Three independent CRISPR edited lines (*CR1*, *CR10, CR13*) harboring distinct mutations in the gene were selected for further characterization (Fig. S17a). For CRISPR edited line *CR1*, 1 bp base was replaced and 8 bp bases were deleted resulting in a truncated protein (11 amino acids total predicted length). The *CR10* and *CR13* mutant lines have a 1 bp deletion and 4 bp deletion resulting in truncated proteins of 21 and 20 aa, respectively. All three lines showed similar delayed fruit ripening initiation of approximately 4 days (Fig. 4a; Fig. S17b) resembling *SlERF.D6* edited mutants (Fig. 2). Consistent with the delayed and slower pigment accumulation phenotype (Fig. S17c), ethylene emission and softening of fruits in the mutants were impaired (Fig. S17d, e). Unlike *SlERF.D6* edited mutants, *SlDEAR2* mutants' locular tissues showed the same color as the WT lines (Fig. S18a). *SlDEAR2* was expressed both in pericarp and locular tissues (Fig. S18b), indicating that the locular color changed in *SlERF.D6* edited mutants may be caused by other factors and the effects of this gene downstream of SlERF.D6 are more prominent in the pericarp.

To gain further insight into the role of SlERF.D6 in regulating the ripening cascade, we also edited the TF *SlTCP12*, a downstream target of SlDEAR2. Its expression pattern during fruit development was similar to that of both *SlERF.D6* and *SlDEAR2* (Fig. S19a). Resulting mutants showed similar delayed ripening initiation (Fig. 4b; Fig. S19b, c) and ethylene emission (Fig. S19d) as mutants in genes encoding its upstream regulators, strongly indicating that this TF is in a direct regulatory chain led by *SlERF.D6*. In summary, we provide novel information describing three TFs working in a sequential ripening regulatory cascade where each is necessary as defined by genetic mutation in normal ripening manifestation.

Furthermore, we can place this regulatory module in the context of additional central and essential ripening regulators and the ripening hormone ethylene.

Discussion

Through mining the fruit spatiotemporal gene expression data (32) and utilizing CRISPR/Cas9 gene editing on multiple candidate genes, we identified several genes with ripening functions including *SlWOX14, SlHsfA6b*, *SlARR11*, and focused on an AP2/ERF family gene, *SlERF.D6*, showing the strongest ripening defects in loss-of-function edited lines. Loss-of-function mutation via CRISPR/Cas9 gene editing, transcriptome and DNA binding (DAP-Seq) analysis, illustrated that *SlERF.D6* is involved in fruit ripening regulation via transcriptional control of a large suite of genes including at least two other TFs, *SlDEAR2* and *SlTCP12*, which also show delayed fruit ripening when functionally analyzed via independent editing of these genes. In addition to those two TFs, *SlERF.D6* influences numerous genes including those contributing to central ripening phenomena of ethylene synthesis and response, carotenoid accumulation, and cell wall and associated textural changes, in addition to a range of additional transcription factor genes. Recent investigations of ripening TFs demonstrates that many are initially expressed in the locule tissue, and mining for putative regulators active in the locule prior to ripening (and later in pericarp), proved a useful route to the identification of novel ripening genes (32, 34).

The AP2/ERF family has members with documented functions in plant hormone and abiotic stress responses (22, 69). In recent years, at least six members (*i.e. LeERF1*, *SlERF6*, *SlERF.B3*, *SlPit4, SlERF.F12* and *SlERF.D7*) of this 140 member gene family in tomato have been linked to plant hormone signaling and fruit ripening (23–29, 70), though none has been analyzed in fruit locule tissues. Examination of the Tomato Expression Atlas (32) showed that four have higher expression in locular tissue than pericarp prior to ripening similar to *SlERF.D6*. CRISPR edited *SlERF.D6* mutants substantially delayed fruit ripening initiation and impaired ethylene production and fruit texture (Fig. 2). The accumulation and constitution of carotenoids were altered in both tissues. In pericarp, reduced lycopene and β-carotene resulted in fruits with less pigment and uneven coloration, while in locule tissue, the most reduced pigments were chlorophyll and lutein with similar or small increases in lycopene and beta-carotene (Fig. S6). Fruits of mutants eventually became red though with less lycopene than WT, demonstrating that while this gene is necessary for normal coordination and initiation of ripening, ripening completion can still eventually be achieved. This reflects a distinct contribution to ripening control as compared to, for example, the *RIN* gene, whose loss results in failure to reach full ripening and suggests either additional genes with compensatory activities that can replace the loss of SlERF.D6 activity or a secondary regulatory circuit.

Transcriptome analysis in WT and *SlERF.D6* mutant fruit revealed pathways of cell wall metabolism, fruit ripening regulation, carotenoids, ethylene, and photosynthesis were primarily influenced (Fig. S7), consistent with a primary role of SlERF.D6 in ripening regulation. In addition to a number of functionally characterized genes (*e.g., PG2a, PL*) (71, 72) in cell wall metabolism, texture and water loss, many additional putative cell wall associated genes were altered in response to *SlERF.D6* mutation (Fig. S8). Fruit ripening regulators along with ethylene associated genes were repressed in early ripening stages

(Fig. S9, S10), and the reduction of ethylene biosynthesis genes *ACS2* and *ACS4* (Fig. S9h, i), as well as ethylene signaling genes including the two predominant ripening-associated ethylene receptors, ETR3 and ETR4 (Fig. S10c, d), likely contributes to the altered ripening response in the mutants. Besides ETR3 and ETR4, ETR6 and ETR7 were reduced in mutants with only slight modifications of the remaining ETRs (Fig. S20). Given the function of ethylene receptor genes as repressors of ethylene phenotypes in the absence of ethylene binding, reduced ethylene receptor levels should elevate ethylene responses (73) and might explain at least in part why *SlERF.D6* deficient fruit eventually achieve WT color and texture phenotypes (Fig. 2).

The photosynthesis-related genes altered with SlERF.D6 loss (Fig. S12) are logically associated with the lower chlorophyll levels in mutant locular tissues (Fig. S6), a tissue where chlorophyll and photosynthetic activity is minimally studied. In pericarp tissues that comprise the flesh of the fruit, the reductions of *DXS, PPH, PSY1, AAT1* and *GF* likely contribute to the decreased accumulation of lycopene and β-carotene in *SlERF.D6* edited mutants (54, 74–77).

Overexpression lines of *SlERF.D6* showed accelerated ripening initiation, earlier ethylene induction and enhanced fruit softening along with curled/epinastic leaves and seedlings displaying the triple response to ethylene (Fig. 3, S13). These phenotypes are consistent with SlERF.D6 playing an important role in ethylene synthesis and signaling.

Analysis of putative SlERF.D6 DNA binding sites revealed many possible targets and combined with parallel transcriptome analysis, suggests that the AP2/ERF transcription factor *SlDEAR2* is a likely *SlERF.D6* target and has furthermore been transcriptionally associated with fruit ripening regulation (67, 68). *SlDEAR2* edited mutants showed the same delayed ripening initiation and impeded coloration phenotypes as *SlERF.D6* mutants but without any alteration of locular pigmentation. The fact that *SlDEAR2* is highly expressed in the locules yet mutants in this gene have no locule phenotype, may suggest the presence of other genes with a complementary function. For example, Solyc02g093130 is the most closely related protein to SlDEAR2 (68% aa identify) and is expressed in the locules at low levels. Whether this gene can compensate for loss of *SlDEAR2* function remains to be determined. Finally, functional analysis of *SlTCP12*, whose promoter is a direct binding target of SlDEAR2, also displayed similar delayed ripening initiation and also without the locular phenotype changes in pigment observed for *SlERF.D6* loss of function. Together these results suggest that *SlDEAR2* is a direct target of SlERF.D6 and is required for a substantial component of SlERF.D6 phenotypic activity except locule pigmentation. Furthermore, *SlTCP12* is a direct target of SlDEAR2 and contributes largely to the effects observed when *SlDEAR2* is mutated.

We present a model that depicts the linear cascade of three transcription factors from distinct gene families in ripening coordination and places them in the context of existing data pertaining to additional regulators (Fig. 4c). On one hand, SlERF.D6 influences ripening initiation by regulating SlDEAR2 and its target SlTCP12 which participates in fruit development with SlTCP21 (Parapunova et al., 2014). *RIN*, *NOR* and *FUL1* are all repressed in *SlERF.D6* mutant lines and *SlERF.D6* is in turn repressed when these genes

are mutated, indicating a feedback interaction with these essential regulators of complete ripening manifestation. It is noteworthy, that as a protein with nucleic acid binding, intrinsically disordered, and prion-like domains, SlERF.D6 is a strong candidate for participation in biomolecular condensate formation. As such, its clear role in regulating ethylene biosynthesis and signaling may be through phase separation, an area of great current interest. The role of SlERF.D6 in locule pigment accumulation, could be direct given the interaction of this TF with multiple carotenoid pathway and pigment accumulation-associated genes, or possibly via functions redundant to SlDEAR2/SlTCP12 or by a currently unknown mechanism.

In conclusion, SlERF.D6 acts as a positive and primary regulator of fruit ripening in tomato, influencing all measured ripening changes and numerous known genes contributing to ripening phenotypes and other central ripening regulators. Knockout of this gene by CRISPR/Cas9 gene editing revealed ripening defect phenotypes in the absence of gene activity that were consistent with largely opposite effects in response to ectopic over-expression. These specifically included delayed ripening and initiation of softening, impaired carotenoid accumulation, and reduced ethylene production and expression of ripening regulatory TF genes. Transcriptome and DAP-Seq together highlight likely primary targets of SlERF.D6 and distinguish genes whose response is downstream in the SlERF.D6 regulatory cascade, providing mechanistic context with known ripening regulators. The structural features of SlERF.D6 further suggests a tantalizing hypothesis that this transcription factor may facilitate ripening and especially ethylene responses via facilitation of liquid-liquid phase separation that enhance the manifestation of ethylene and ripening activities.

Materials and Methods

Plant materials. Wild-type (*Solanum lycopersicum* cv. Ailsa Craig) seeds were obtained from the Tomato Genetics Resource Center (TGRC, https://tgrc.ucdavis.edu/). Fruits were tagged upon reaching 1 cm in size and the number of days to Breaker was determined. AC and mutant fruits reach 1 cm approximately 7 days following pollination. Fruits were collected on a series of dates defined as DPA (day post anthesis) where each date indicates the time from tagging at 1 cm fruit diameter plus 7 days. All plants were grown in greenhouses at the Boyce Thompson Institute (Ithaca, NY) with 16-hour light (27°C) and 8-hour dark (15°C) cycle.

Metabolite and Molecular Analysis. Details of ethylene and texture measurements, carotenoid extraction and quantification, DNA constructs, tomato transformation, transcriptome analysis, qRT-PCR, DNA sequencing, Illumina read processing, GO enrichment analysis, and DAP-Seq analyses are provided in the Supporting Information. All primers used in this work are listed in Dataset S8.

Declarations

Data Availability

Raw RNA-Seq and DAP-Seq reads have been deposited in the NCBI Bioproject database under the accession number **PRJNAXXXX**.

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Author contributions

JJG, YC planned and designed the research; YC performed the research; YC, ZF, XW, VC, TF, TT analyzed data; JY assisted with DAP-Seq; YC, JJG wrote the manuscript; ML, YL, ZY assisted with thoughtful insights, suggestions and edits to improve the manuscript content and structure.

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Figures

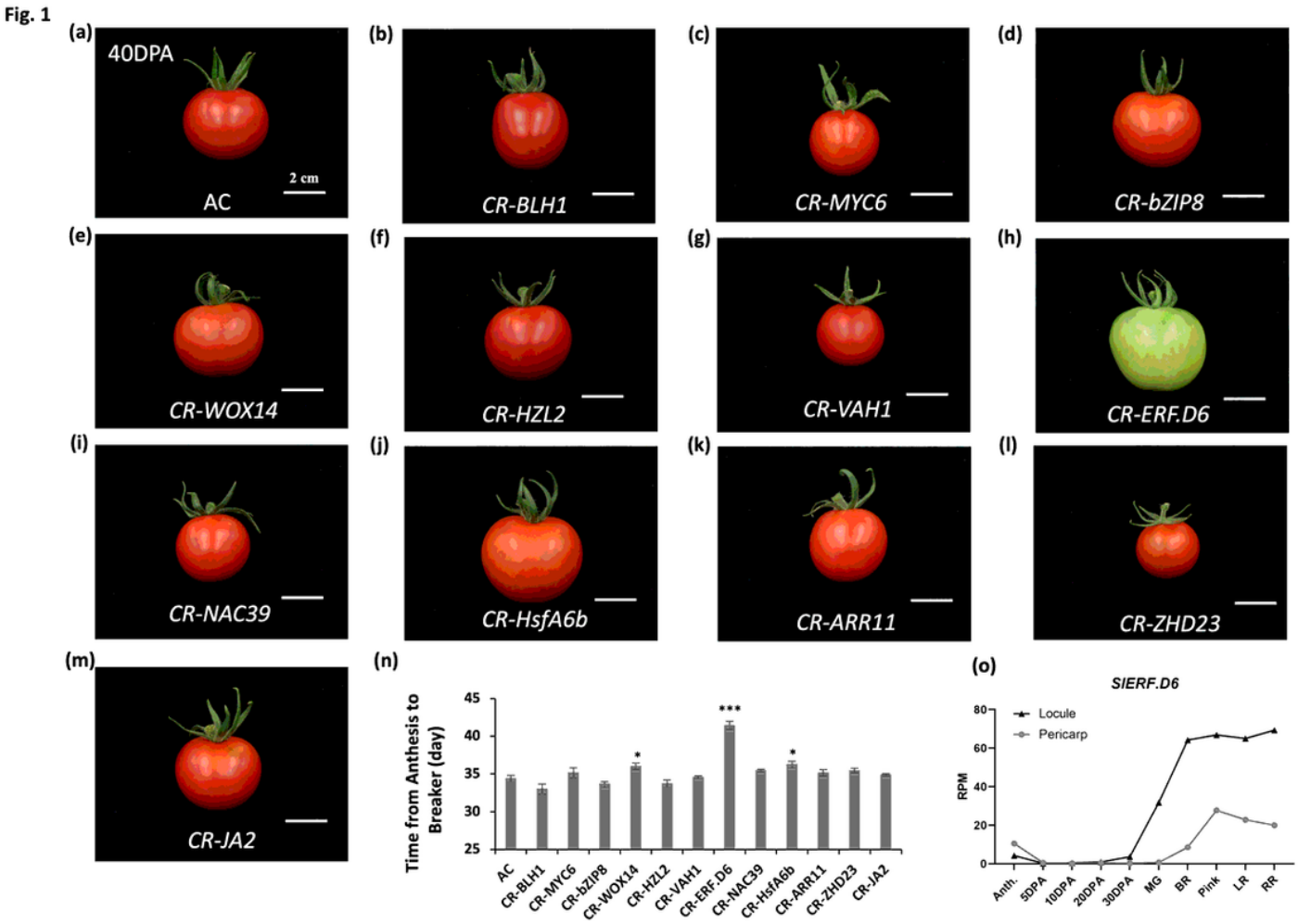

Figure 1

Images and ripening onset of CRISPR/Cas9 tomato mutants.

(a-m) Representative fruit phenotypes of **(a)** *Ailsa Craig* WT and edited mutants of 12 TFs at 40 DPA. **(b)** *CR-BLH1*, Solyc01g007070; **(c)** *CR-MYC6*, Solyc01g102300; **(d)** *CR-bZIP8*, Solyc01g102300; **(e)** *CR-WOX14*, Solyc02g082670; **(f)** *CR-HZL2*, Solyc02g087840; **(g)** *CR-VAH1*, Solyc03g113270; **(h)** *CR-ERF.D6*, Solyc04g071770; **(i)** *CR-NAC39*, Solyc05g007770; **(j)** *CR-HsfA6b*, Solyc06g053960; **(k)** *CR-ARR11*, Solyc08g077230; **(l)** *CR-ZHD23*, Solyc09g065670; **(m)** *CR-JA2*, Solyc12g013620. **(n)** Time from anthesis to the breaker stage of *Ailsa Craig* WT (AC) and edited mutants. Statistical significance was determined by Student's t-test: *, 0.01 < P < 0.05; ***, P < 0.001. Error bars indicate standard error (SE). **(o)**Expression pattern of *SlERF.D6* in tomato locule and pericarp using data from the Tomato Expression Atlas (https://tea.solgenomics.net/).

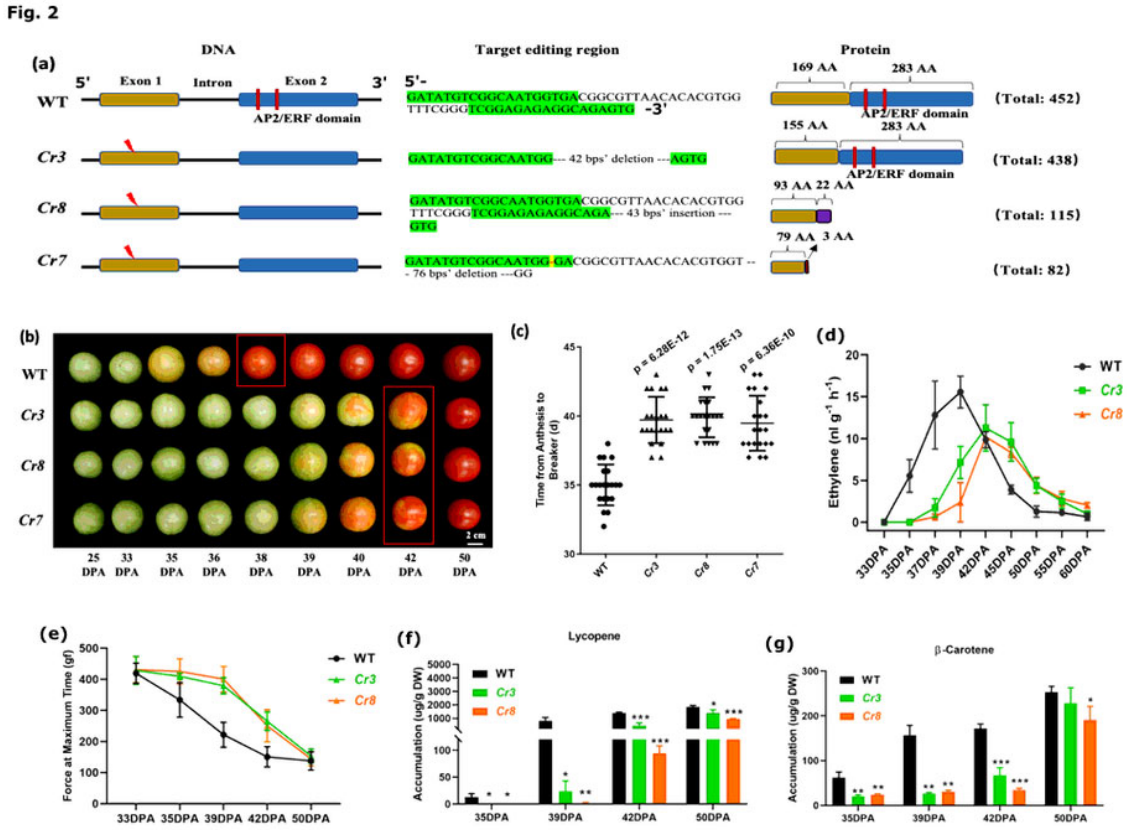

Figure 2

***SlERF.D6*gene-edited mutations and phenotypes.**

(a) Gene models of WT and edited *SlERF.D6* alleles in homozygous edited lines with lightning bolts indicating the position of edits (left), linear DNA sequences of CRISPR target region with green highlighted gRNA sequences in the WT and mutant alleles (middle), and resulting hypothetical protein

products with novel amino acid sequences in purple (right). For wild-type (WT), a full-length protein (452 amino acids) is predicted. For edited lines, predicted truncated proteins of the indicated lengths are shown in parenthesis. **(b)**Time course of fruit development from WT and CRISPR edited mutants. **(c)**Time from anthesis to the breaker stage (Br) in WT and CRISPR edited lines. **(d)**Ethylene production **(e)**, fruit firmness **(f)**, lycopene and **(g)**β-carotene accumulation in WT and CRISPR edited fruits at the indicated DPA. Statistical significance was determined by Student's t-test: *, 0.01 < P < 0.05; **, 0.001 < P < 0.01; ***, P < 0.001. Error bars indicate standard error. DPA, days post anthesis.

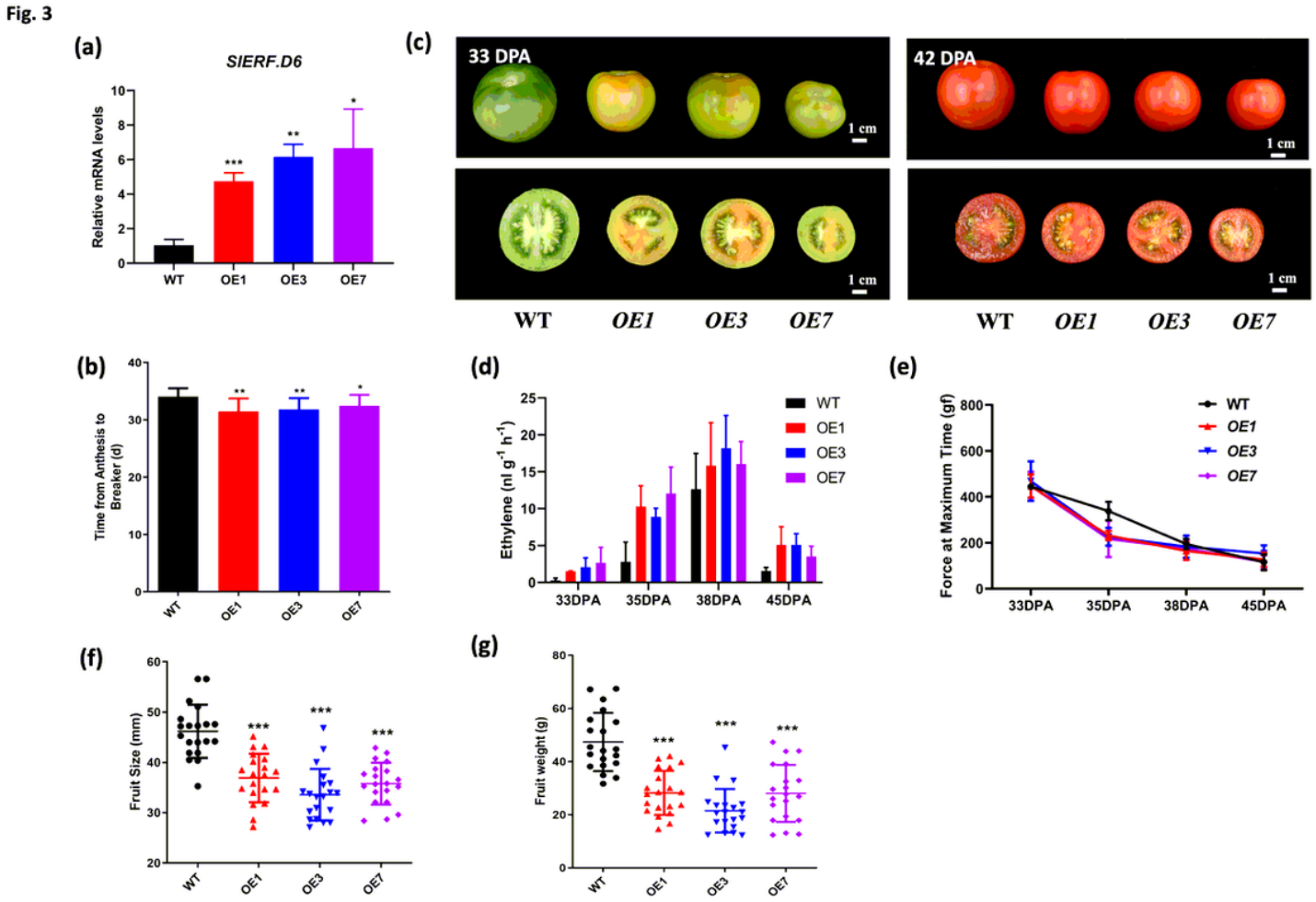

Figure 3

Phenotypes of *SlERF.D6*over-expression lines.

(a) Transcript abundances of *SlERFD6* assessed by qRT-PCR in fruits of wild type (WT) and *SlERF.D6-OE* lines at the breaker stage. **(b)** Time in days from anthesis to breaker in WT and *SlERF.D6-OE* lines. For each line, n ≥ 15. **(c)** Representative fruits of WT and *SlERF.D6-OE* lines at 33 DPA and 42 DPA. **(d)** Ethylene production and **(e)**fruit firmness of WT and *SlERF.D6-OE* fruits at the indicated DPA. **(f)**Fruit size (diameter in mm) and **(g)** weight of WT and *SlERF.D6-OE* fruits at 7 days post breaker stage (Br+7).

Statistical significance was determined by Student's t-test: *, 0.01 < P < 0.05; **, 0.001 < P < 0.01; ***, P < 0.001. *OE1, OE3,* and *OE7* are three independent *SlERF.D6*overexpression lines. Vertical bars represent SE.

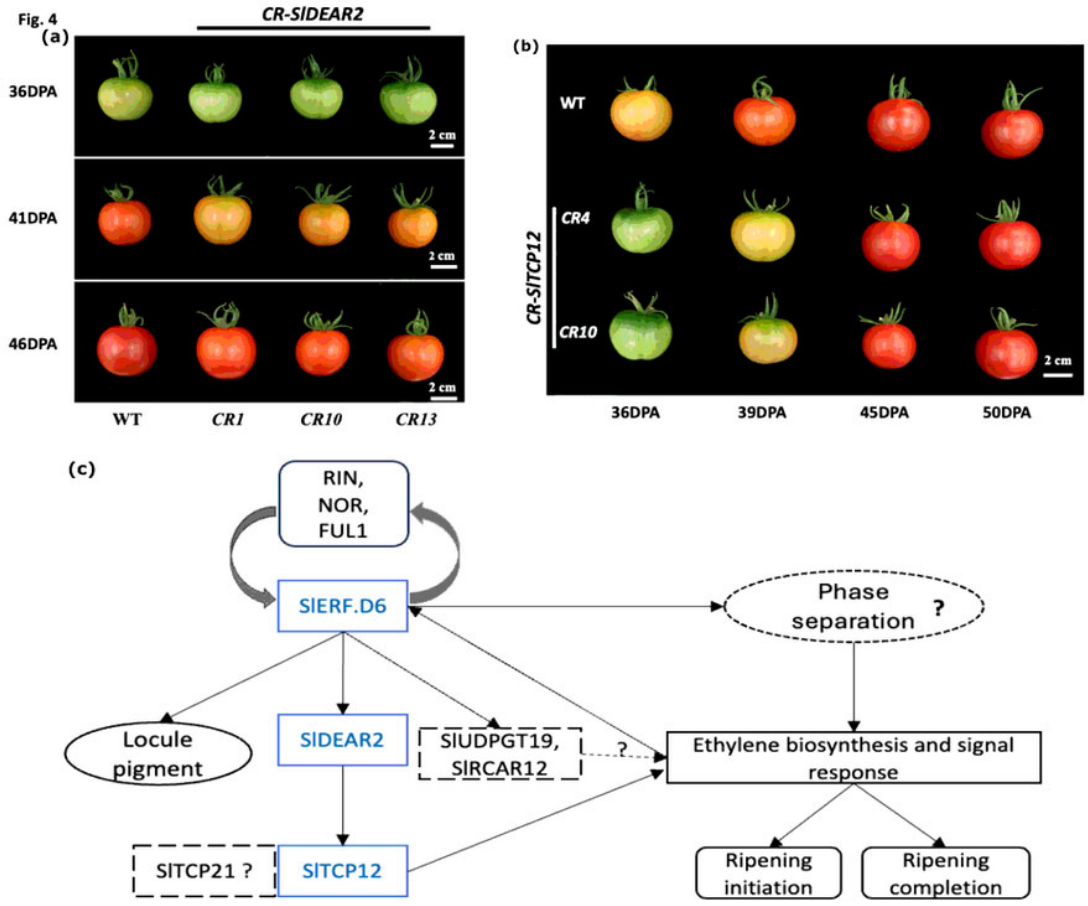

Figure 4

SlERF.D6, SlDEAR2, SlTCP12 module mediating ripening regulation.

Delay of tomato fruit ripening initiation in CRISPR/Cas9 edited lines of the **(a)** *SlDEAR2* and **(b)** *SlTCP12* genes. Fruit from three and two independent edited lines are shown for *SlDEAR2* and *SlTCP12*, respectively, at the indicated DPA. **(c)** SlERF.D6 mediated ripening regulatory network. SlERF.D6 is a newly defined component of a regulatory module including SlDEAR2 and SlTCP12 that is necessary for normal ripening initiation and progression. The *SlERF.D6* promoter interacts with the RIN, NOR and FUL1 ripening regulators and is reduced in expression when the corresponding genes are mutated or repressed. *RIN*, *NOR*and *FUL1* expression levels are in turn influenced by *SlERF.D6*activity indicating bidirectional regulation. SlTCP21 may interact with SlTCP12. The SlERF.D6 module influences ethylene synthesis and expression of multiple ethylene signal transduction genes including fruit-associated ethylene receptors. The presence of multiple intrinsically disordered domains in the SlERF.D6 peptide suggests the intriguing

possibility that this regulator may function through formation of localized liquid/liquid phase separations.

Supplementary Files

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