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Article

Fruit ripening and postharvest changes in very early–harvested tomatoes

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Abstract

It is well known that if a fruit is harvested extremely early its development and function are interrupted, and it may never attain full maturity and optimal quality. Reports revealing insights regarding the alterations of maturation, ripening and postharvest quality in very early picked fruits are rare. We examined the effects of early harvesting on tomatoes by characterizing different accessions at the molecular, physiological, and biochemical levels. We found that even very early–harvested fruits could achieve postharvest maturation and ripening though with some defects in pigment and cuticle formation, and seeds from very early–harvested fruits could still germinate and develop as normal and healthy plants. One critical regulator of tomato cuticle integrity, *SlCER1–2*, was shown to contribute to cuticle defects in very early–harvested fruits. Very early fruit harvest still allowing ripening and seed development indicate that the genetic and physiological programs of later maturation and ripening are set into motion early in fruit development and are not dependent on complete fruit expansion nor attachment to the plant.

Introduction

Fleshy fruit maturation ends with ripening and involves regulated developmental changes in chemistry, metabolism and physiology. Ripening can include changes in nutritional compounds, synthesis and accumulation of characteristic pigments, textural modifications, and altered aroma volatiles of seed bearing carpel or adjacent floral tissues [1–5]. Physiologically, fleshy fruits are described as climacteric or non-climacteric according to whether or not they exhibit an increase in respiration and ethylene production, respectively, at the onset of ripening [6, 7]. While climacteric fruits, such as tomato, display the increases in respiration and ethylene at the onset of ripening, many non-climacteric still may respond to applied ethylene [8]. Significant strides have been made in understanding the ripening regulatory mechanisms of climacteric fruit maturation, often via studies in tomato (*Solanum lycopersicum*), the model crop for many fleshy fruit studies [2, 9–11]. Even in tomato little research has explored maturation, ripening and water loss of very early–harvested (VEH) fruits which can have relevance to profitable production systems, seed production, breeding strategies and as a means to more fully understand fruit maturation. Climate change, drought, flooding, wildfires, and biotic stress cause hundreds of billion dollars of losses around the globe (https://www.fao.org/resources/digital-reports/disasters-in-agriculture/en/). Better understanding of early harvest fruit development and maturation could help address crop losses and provide alternatives for successfully securing seed during breeding and for biological inquiries.

Growers often have to harvest fruits even though some have not fully developed, for example, when destructive machine-assisted harvesting is the only economical route. Those VEH fruits can be discarded as invaluable because it is often thought that they would not ripen at all. By examining fruit ripening indexes and molecular traits of VEH tomato fruits over extended storage periods, we observed that while fruit expansion arrests with harvest, development and ripening proceed even in VEH fruits and often with few defects except for extremely early harvested fruit where abnormal cuticle formation and increased water loss occur. Here we demonstrate that a regulator of cuticle formation, *SlCER1–2*, is critical in VEH fruit water loss. *SlCER1–2*, a homolog of *Arabidopsis thaliana ECERIFERUM1* (*CER1*), is a central gene in the biosynthesis of cuticle waxes and specifically, very-long-chain (VLC) alkanes [12]. By using CRISPR-Cas9 editing technology, edited *SlCER1–2* loss-of-function tomato lines were generated and presented defective fruit cuticle phenotypes consistent with observed postharvest water loss.

Results

Very early–harvested fruits reached maturity but with some alterations

Tomato fruits of different accessions, including *S. lycopersicum* cvs. Ailsa Craig, M82, Micro-Tom, TS-272 (a modern variety also known as 174 MS), TS-665 (an heirloom variety named Taxi) and *Solanum pimpinellifolium* (BGV007149), were harvested at multiple stages and left to mature on the same lab bench for 42 days (Fig. 1a-c) when most fruits reached visual maturity as indicated by the ripening initiation time (from anthesis to breaker stage) measured from on-vine fruits (Fig. S1). Most fruits accumulated carotenoids

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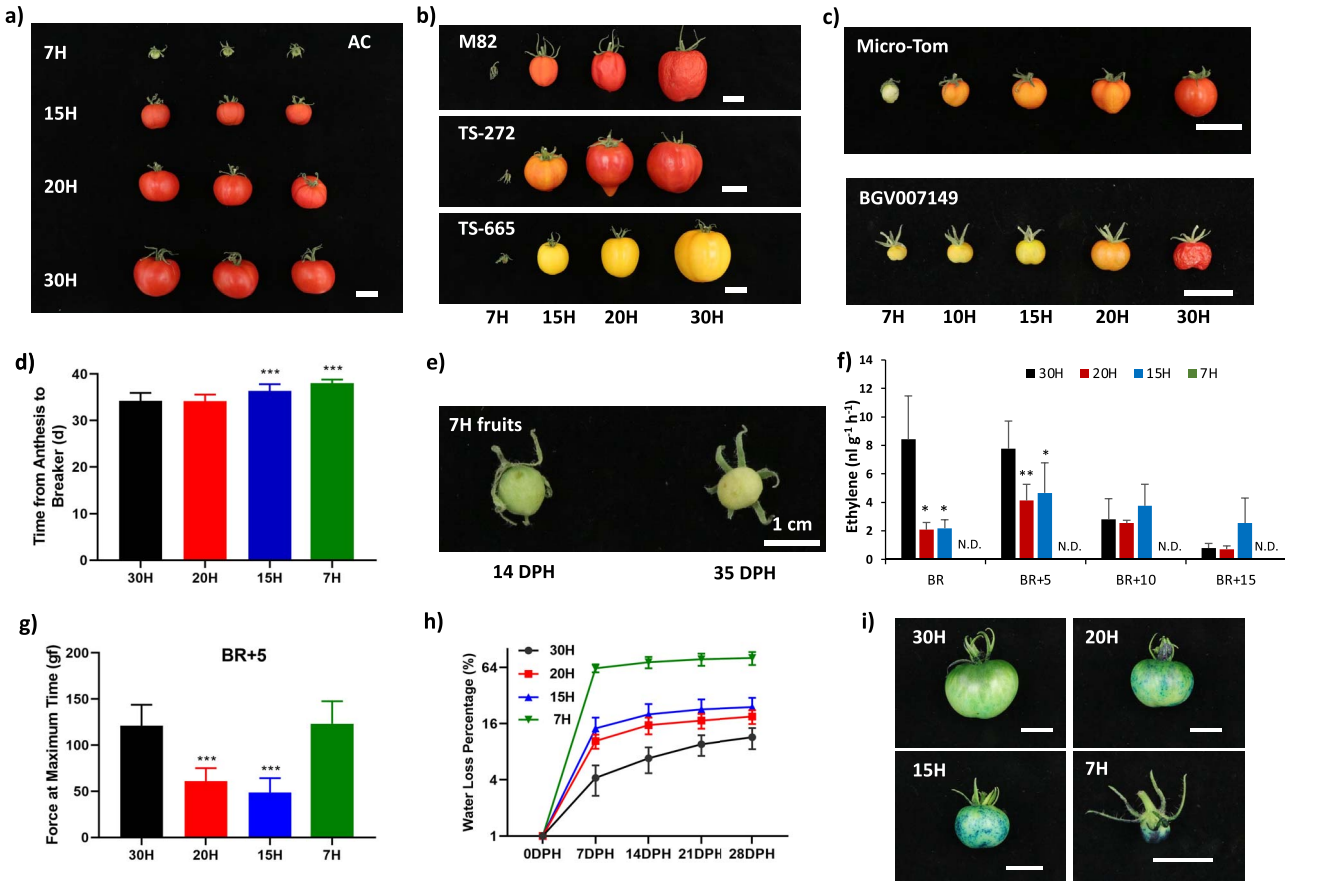

Figure 1. Very-early-harvest fruits reached maturity. (a-c) Tomato fruits of different accessions (*Solanum lycopersicum* cvs. Ailsa Craig [AC], M82, TS-272, TS-665, Micro-Tom, and *Solanum pimpinellifolium* BGV007149) were harvested at indicated stages (7H, 10H, 15H, 20H, and 30H corresponds to 7, 10, 15, 20, and 30 DPA-harvested) and left on the same lab bench for 42 days. Panel "a" shows three replicate fruits for each harvest stage. (d) Time period from anthesis to the breaker stage of 30H and very early harvested (VEH) fruits at the indicated harvest ages. (e) Color alteration observed on 7H fruits. (f) Ethylene production in 30H and VEH fruits at different ripening stages. (g) Fruit firmness in 30H and VEH fruits at the BR + 5 (5 days after breaker) stage. (h) Water loss percentage of VEH fruits. Fruits were harvested at the indicated stage and kept at room temperature for 4 weeks. The weight loss per fruit was calculated every 7 days. Values represent means ± SE ($n \geq 6$). (i) Toluidine Blue (TB) solution staining of fruits. Fruits were harvested at indicated stages and stained with 1% TB solution for 18 hrs. White scale bar corresponds to 2 cm. DPH, days post harvest. N.D., not detected. Asterisks indicate statistical significance using Student's *t*-test: *$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$.

as normal, but color defects were observed on the least mature fruits harvested between 7 and 20 days post anthesis (DPA). In general, the earlier the harvest, the more severe the deviation for normal development and ripening. Seven DPA-harvested (7H) fruits accumulated low levels of carotenoids, and in some cases desiccated (Fig. 1b, Fig. S2a–f). Desiccation was dependent on the fruit size, with fruits under 1 cm generally more likely to dehydrate (Fig. S2g). We note that TS-665 contains the *r* gene (phytoene synthase loss-of-function mutation) and is yellow and carotenoid-deficient even when matured on the vine. Micro-Tom and BGV007149, the earliest harvest fruits do not turn full red even when we have kept them as long as 90 days. Micro-Tom harbors multiple mutations including hormone (brassinosteroid) synthesis which itself can influence pigment accumulation, making such phenotypes difficult to interpret. BGV007149 is a small fruited tomato wild ancestor, *S. pimpinellifolium*. We have not measured carotenoids in these accessions though would suspect both are accumulating beta-carotene at the expense of lycopene in all but the 30H fruit.

In order to better understand fruit development and maturation in VEH fruits, we initially analyzed fruit development in VEH Ailsa Craig tomato fruits harvested at 7, 15, 20, and 30 DPA where harvests at 20 DPA and earlier yielded smaller fruit (Fig. 1a). Ripening initiation was delayed in 15 DPA-harvested (15H) and 7H fruits (Fig. 1d). All Ailsa Craig fruits attained visual red coloration except 7H fruits which matured to light yellow by 35 DPH when the experiment ended (Fig. 1e). Quantitative data on pigment abundance showed similar levels of the major carotenoids (lycopene and beta-carotene) and some changes in the minor level precursor compounds among fruits at the breaker+5 stage (Fig. S3). Ethylene production was reduced in 20 DPA-harvested (20H) and 15H fruits where peak ethylene was also reduced and the decline in ethylene associated with later ripening was also extended. Ethylene was not detected in 7H fruits (Fig. 1f). Fruit firmness was reduced in 20H and 15H fruits but did not change in 7H fruits (Fig. 1g). Water loss increased in all VEH fruits (Fig. 1h), likely due to deficient cuticle deposition as assessed by Toluidine blue staining (Fig. 1i).

In addition to altered ripening parameters, seed numbers were substantially decreased in VEH fruits (Fig. 2a). Seed size in 7H and 15H fruits was notably smaller than later harvested fruits (Fig. 2b). Although even with reduced size, seeds from VEH fruits at all stages including 7H maintained very high germinate rates (Fig. 2b) and presented normal development and growth that were indistinguishable among harvest dates (Fig. 2c–d).

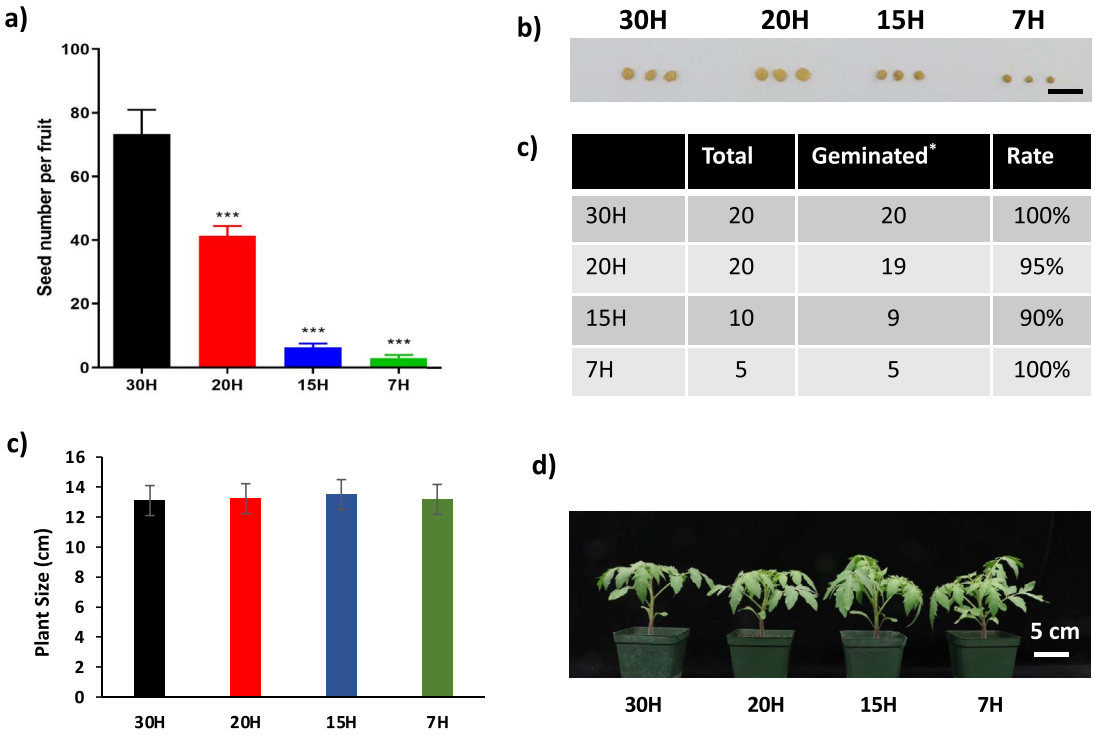

Figure 2. Phenotypes of seeds and plants (a). Seed number in 30H and VEH fruits (Ailsa Craig). Asterisks indicate statistical significance using Student's t-test: ***$P < 0.001$. (b) Seed phenotype and germination rate. Black bar means 1 cm. *, Putting seeds on the plates after 7 days. (c) Size of plants germinated from seeds of 30H and VEH fruits. (d) Plants of the next generation. Plant sizes were measured and photos taken at the 4-week-stage.

To determine whether ripening in VEH fruits was influenced by the developing seeds, we induced parthenocarpy by manually removing stamens before flower fertilization and treating with dichlorophenoxyacetic acid (2,4-D) and gibberellic acid (GA3) [13]. The VEH parthenocarpic fruits showed the same ripening phenotype as seeded VEH fruits (Fig. S4), indicating that maturation and ripening in VEH fruits is developmentally separable from seed development.

Molecular modifications in VEH fruits revealed by transcriptomic analysis

To better understand the molecular changes occurring during the development and maturation of VEH fruits, we performed RNA-Seq transcriptome analyses on 30H, 20H, and 15H fruits. Five stages of fruit development were analyzed for 30H fruits: MG, mature green; BR, breaker; BR + 5, 5 days after breaker; BR + 10, 10 days after breaker; BR + 15, 15 days after breaker, in addition to BR + 5 20H and 15H fruits so as to assess where in the spectrum of maturation the VEH fruits fell relative to the 30H reference (Fig. 3a).

Principal component analysis (PCA) clearly showed that all five stages in 30H fruits could be separated while all BR + 5 stages of 30H, 20H and 15H fruits were clustered (Fig. 3b). This result suggests that at the level of transcriptome activity, VEH fruits behaved similarly in terms of reaching a comparable maturation status irrespective of harvest time, although the number of DEGs (fold change ≥2, adjusted $P < 0.5$) between 15H and 30H was greater than that between 20H and 30H (Fig. S5) suggesting that while similar in development and transcriptional activity they are not identical. Gene ontology (GO) enrichment analysis showed that the most enriched up-regulated GO terms were cellular anatomical and seed maturation in 15H and 20H fruits, respectively (Fig. S6a, b). The top downregulated GO term was rhythmic process in both 15H and 20H fruits (Fig. S6c, d). And other interesting downregulated GO terms that were enriched in 15H fruit were fruit ripening, ethylene biosynthesis, and ethylene metabolic processes (Fig. S6c), which in some instances corresponded with alterations in measured traits of ripe fruits (Fig. 1d-g).

Dozens of ripening regulators have been identified to date (Supplemental Data S1) [2, 9, 14–44], and of the thirty-one analyzed, four including *Lutesent2*, *SlMYB70*, *SlPP2C3*, and *SlERF.D7* were significantly changed in gene expression in 15H compared with 30H fruit (Fig. 3c). *Lutesent2* positively influences tomato chloroplast development and ripening, while *SlMYB70* and *SlPP2C3* suppress maximal ripening progression [32, 43]. The promotive ripening regulator *SlERF.D7* [31] was upregulated in 15H fruit, suggesting a possible complementary mechanism that might counter the effects of elevated ripening repressors in 15H fruit.

Examining genes participating in ethylene biosynthesis and signaling (Supplemental Data S2), we observed that *ACS2* and *ACS4*, encoding a rate limiting step in ethylene synthesis, were greatly repressed in 15H BR + 5 fruits, while expression of *ACO1* and *ACO5*, also necessary for ethylene synthesis, were reduced in both 20H and 15H BR + 5 fruits (Fig. 3d) and consistent with the reduced ethylene synthesis. As for signaling genes, changes were minimal and balanced in each signaling component family, suggesting little if any effect on this aspect of ethylene responses (Supplemental Data S2). The reduction of ethylene evolution in VEH fruits (Fig. 1f) is thus most likely due to reduced expression of ethylene synthesis genes [45].

Fruit firmness related genes were also altered in expression in VEH fruits (Supplemental Data S3). Consistent with enhanced softening phenotypes of 15H and 20H fruits, one transcriptional regulator of locule liquefaction, *MBP3* [46, 47], was significantly upregulated, though four cell wall enzymes whose reduced expression would be anticipated to associate with increased

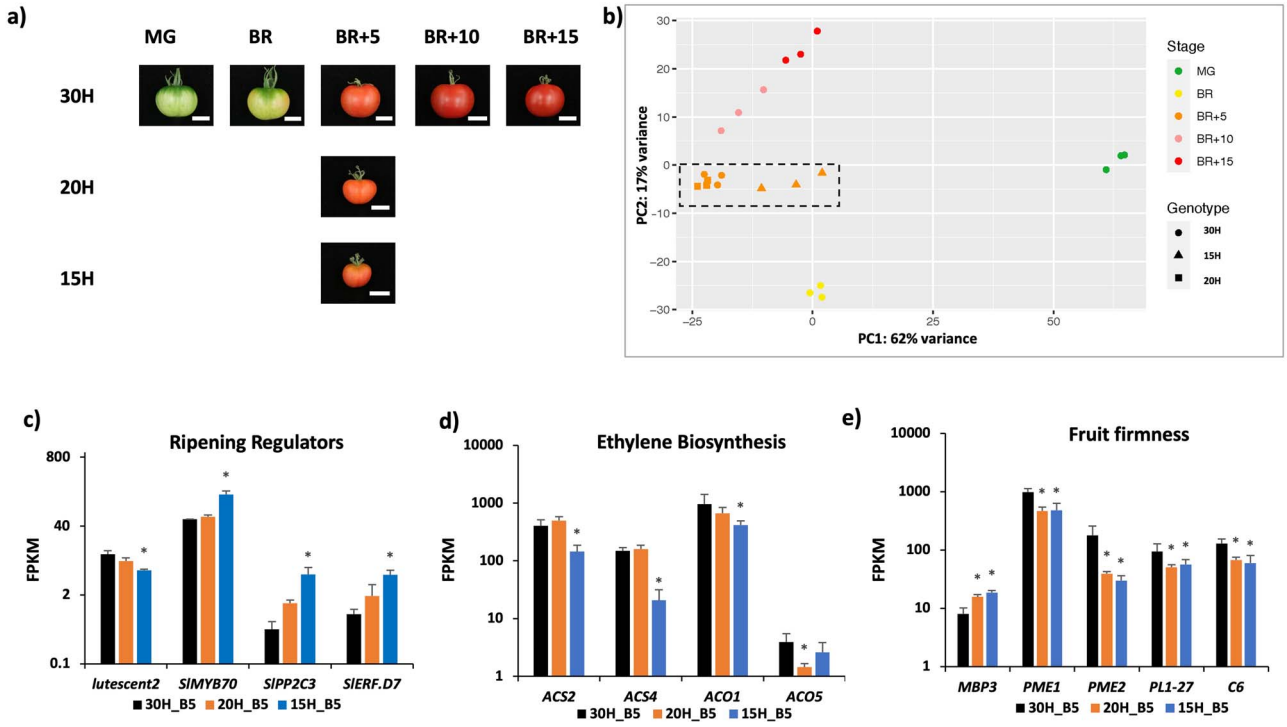

Figure 3. RNA-Seq profiling of VEH fruits. (a) Illustration of fruit samples (Ailsa Craig) for RNA-Seq. MG, mature green; BR, breaker; BR + 5, 5 days after breaker; BR + 10, 10 days after breaker; BR + 15, 15 days after breaker. White bars = 2 cm. (b) Principal component analysis (PCA) plot representing transcriptome profiles of VEH and 30H fruits. (c) Four ripening regulators were significantly changed in 15H fruits at the BR + 5 stage. (d) Four ethylene biosynthesis genes were changed in VEH fruits. (e) Five fruit firmness associated genes were greatly influenced in VEH fruits. FPKM, fragments per kilobase transcripts per million mapped fragments.

softening, *PME1*, *PME2*, *PL1–27*, and *C6* [48, 49], were repressed (Fig. 3e). This might suggest that enhanced softening of 15H and 20H fruit was associated with earlier locule liquefaction. In addition, thinner pericarps were observed in VEH likely also contributing to enhanced softening phenotypes (Fig. S7). Finally, because the time for VEH fruits to reach BR + 5 stage is longer than for 30H fruit, more water is lost in VEH fruit which is an additional likely contributor to textural changes.

CER1–2 is central in the formation of the cuticle in VEH fruits

Transcriptome analysis of cuticle associated genes revealed eight that were upregulated in VEH fruits while these fruits in turn were cuticle deficient (Fig. 4a, Supplemental Data S4). Inhibition of *CD2* (*Cutin Deficient 2*) causes defects in cuticle function and structure changes in tomato fruit [50]. Knockdown of *CER1–1* (*ECERIFERUM 1–1*) reduced wax alkane production and caused increased water loss in transgenic fruits [12]. Tomato *slcer6* mutation reduced cuticular wax biosynthesis and increased water loss [51]. Although *CER1–5* and *CER3–1* have not been functional identified, they were also predicted to have positive associations with tomato cuticle formation [12]. Knowledge of lipid transfer protein (LTP) function in cuticle formation is limited, but they have been suggested to contribute to cuticle formation [52, 53]. While these genes are generally associated with cuticle deposition, the fact that they were elevated may indicate that concerted misregulation of cuticle-associated genes has negative consequences on cuticle formation or their upregulation may alternatively reflect a compensatory response to other alterations in cuticle deposition resulting from VEH. *CER1–2* was the only cuticle synthesis gene downregulated in our analysis (Fig. 4b).

We searched for any alterations of additional cuticle-associated genes including four transcription factors, *SlMIXTA-like*, *SlSHN1*, *SlSHN2*, and *SlSHN3* [54–57]. None had significantly altered expression in VEH fruits (Supplemental Data S4). *SlCER1–2* was the only downregulated regulatory gene of cuticle synthesis and thus may be essential to cuticle formation during fruit development. *SlCER1–2* has recently been shown to be regulated by SlCNR associated with postharvest water loss of ripening fruit [58].

To better understand the function of *CER1–2* in tomato fruit cuticle formation and fruit development, we generated CRISPR/Cas9 edited mutations at the *CER1–2* locus in tomato cultivar Ailsa Craig. Two independent CRISPR edited (CR) lines (*CR1*, *CR25*) harboring distinct mutations in the gene were selected for further characterization (Fig. 4c). Both mutations represent predicted early translation termination of the *CER1–2* transcript and thus are likely loss-of-function mutations, further supported by the similar phenotypes displayed by both lines. Toluidine blue staining confirmed that cuticle integrity was impaired in CR lines (Fig. 4d) and water loss increased in mutant fruit compared with wild type when stored at room temperature (Fig. 4e, f). We show that alteration of CER1–2 influences fruit cuticle integrity and water loss and because of its repression in VEH fruit, likely contributes to the altered cuticle and water loss phenotypes observed in these fruits. Besides of its function in cuticle formation, we also investigated its role in fruit ripening because it specially expresses during the fruit ripening process (Fig. S8a, b). Neither ripening initiation nor ripening completion time was changed in CR lines (Fig. S8c, d), suggesting its exclusive role in fruit cuticle formation and water loss. Together these results confirm the previously hypothesized role of CER1–2.

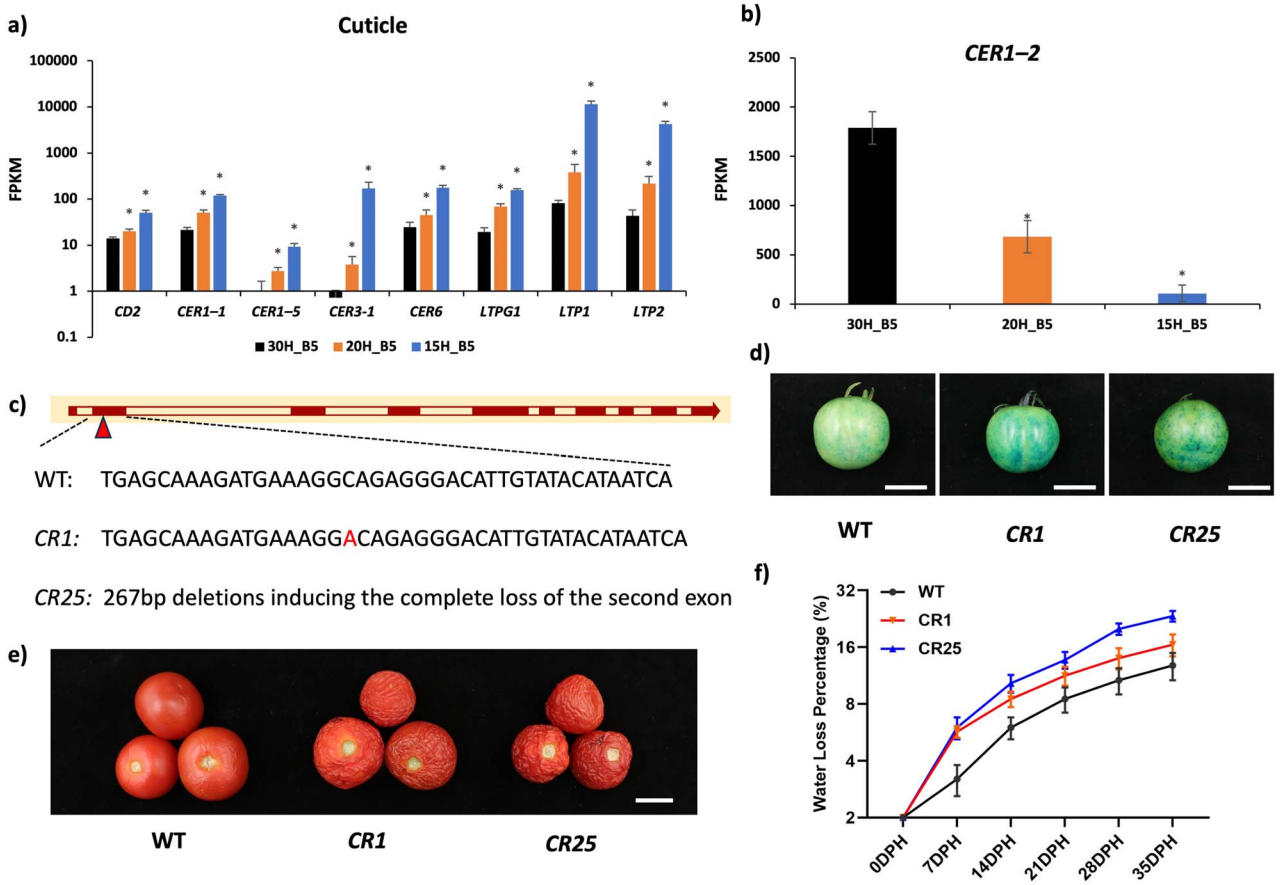

Figure 4. A master regulator for the formation of fruit cuticle. (a) Genes contributing to cuticle formation were upregulated in cuticle deficient VEH fruits (Ailsa Craig). (b) *CER1–2* was the only downregulated cuticle related gene in VEH fruits. (c) CRISPR edited information of *CER1–2*. Red triangle points to the target sequence of the gRNA. (d) Toluidine Blue solution staining of fruits. Fruits were harvested at MG stage and stained with 1% TB solution for 18 hours. (e) Fruits harvested and stored at room temperature after 6 weeks. (f) Water loss of fruits in 5 weeks post harvest. White bar corresponds to 2 cm.

Conclusion

Our study shows that VEH fruits displayed modest effects on fruit ripening in terms of color and water loss, especially at the earliest harvest times, while transcriptome analysis indicated that molecular alterations on ripening related factors in VEH fruits are minimal and consistent with the observed phenotypic differences. Indeed, even fruits harvested at under 25% (7H) of a typical on vine maturation time for Ailsa Craig (30H/MG) yielded viable seed though at reduced numbers. While these results suggest the potential for very early harvest, quality effects on specific crops and genotypes would still need to be undertaken to determine practical value. While the transcriptome analysis of the Ailsa Craig fruit examined here suggest only modest changes in response to VEH, M82 tomato fruit harvested at the mature green and breaker stages did present lower levels of sucrose, carotenoids, malate, and other amino acids [59]. Even if reduced in quality, VEH tomato fruits could have uses in processed foods such as plant-based meat alternatives [60] for enhanced moisture, fiber, and color attributes.

Very early harvested fruits have substantial changes in fruit texture and water loss during the storage due to impaired fruit cuticle integrity, yet produce viable seed which has important implications to the seed industry, plant breeding, and breeding strategies. The fact that fruit can develop and mature absent seed set is not surprising given the prevalence parthenocarpic fruit varieties driven by consumer preference. Nevertheless, the observation that fruit maturation and ripening is independent of seed development in VEH fruit was not necessarily anticipated and had not been previously addressed to our knowledge. The separation of seed development from fruit maturation in parthenocarpic VEH fruit suggests that maturation programs are set very early in development and are confined to the fruit organ. Practically, VEH may be applicable to some situations involving parthenocarpic fruit as well as seeded fruit, possibly limited to processed applications, depending upon the potential for quality alteration. Reduced expression following VEH and gene editing of the *CER1–2* gene as a means of assessing function demonstrates that impaired cuticle integrity and increased water loss after harvesting in VEH fruits are due at least in part to reduced expression of *CER1–2*. While this analysis provides insights into the mechanisms of altered fruit development in VEH fruits, it also suggests that identification and selection of *CER1–2* alleles with higher expression and/or targeted engineering of this gene may prove useful in developing more climate and postharvest resilient crops.

Materials and methods

Plant materials

Tomato (*S. lycopersicum*) seeds were acquired from the University of Florida (H. Klee/D. Tieman lab) and the Tomato Genetics Resource Center (TGRC; https://tgrc.ucdavis.edu/). Fruits were

tagged at the anthesis stage and collected on recorded DPA (days post anthesis). All plants were grown in greenhouses at the Boyce Thompson Institute (Ithaca, NY) under a 16-hour light (26–29°C) and 8-hour dark (15–18°C) cycle.

Generation of transgenic tomato plants

A gRNA targeting *SlCER1–2* was designed utilizing CRISPR-P (version 2.0, http://crispr.hzau.edu.cn/CRISPR2) [61].The gRNA cassette was cloned into a binary vector p201N:Cas9 by Gibson assembly [62]. Colonies harboring the accurate gRNA sequence were validated through PCR and Sanger sequencing. Tomato transformation and the screening of gene-edited lines were performed as previously described [63]. All primers used in this work are listed in Supplemental Data S5.

Carotenoid, ethylene, and fruit texture measurements

Carotenoids, ethylene, and firmness measurements were performed as previously described [63]. For ethylene measurement, at least three VEH fruits were held together in a closed container counted as a single biological replicate. For all measurements, at least three biological replicates were performed.

RNA-Seq library construction and sequencing

RNA was extracted from tomato tissues grounds to a powder in liquid nitrogen, and RNA-Seq libraries were constructed using protocols described previously [64, 65]. Paired-end DNA sequencing was performed using the Hiseq X platform (Illumina) at Psomagen, Inc. (MD, USA).

RNA-Seq data processing and analysis

RNA-Seq data processing and analysis were performed following the methodologies outlined in previous studies [63, 66–68]. In brief, raw RNA-Seq reads were downloaded from the Psomagen website trimmed for vector and primer sequences, filtered for any non-tomato contaminants and then aligned to tomato reference genome SL4.0 [69] with ITAG4.1 gene models. Raw counts in each gene model were normalized to generate FPKM (fragments per kilobase transcripts per million mapped fragments) values and to identify DEGs (adjusted $P < 0.05$ and an absolute Log_2 ratio ≥ 2). GO term enrichment analysis was performed using Blast2GO [68]. Sequencing statistics and sample correlations are shown in Supplemental Data S6.

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Author contributions

Y.C., J.J.G. planned and designed the research. Y.C. performed the research. Y.C., X.T., Z.F., analyzed the data. Y.C., J.J.G. wrote the manuscript.

Data availability

Raw RNA-Seq reads have been deposited in the NCBI BioProject database under the accession number PRJNA1102113.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Supplementary data

Supplementary data is available at *Horticulture Research Journal* online.

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