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FEATURED ARTICLE

Combined transcriptome, genetic diversity and metabolite profiling in tomato fruit reveals that the ethylene response factor *SlERF6* plays an important role in ripening and carotenoid accumulation

Je Min Lee[1], Je-Gun Joung[1], Ryan McQuinn[2], Mi-Young Chung[1], Zhangjun Fei[1,2], Denise Tieman[3], Harry Klee[3] and James Giovannoni[1,2,*]

[1]*Boyce Thompson Institute for Plant Research, Tower Rd., Cornell University campus, Ithaca, NY 14853, USA,*
[2]*USDA-ARS Robert W. Holley Center for Agriculture and Health, Tower Rd., Cornell University campus, Ithaca, NY 14853, USA, and*
[3]*Horticultural Sciences Department, University of Florida, Gainesville, FL 32611-0690, USA*

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*For correspondence (fax +1 607 254 1242; e-mail jjg33@cornell.edu).

SUMMARY

Solanum lycopersicum **(tomato) and its wild relatives harbor genetic diversity that yields heritable variation in fruit chemistry that could be exploited to identify genes regulating their synthesis and accumulation. Carotenoids, for example, are essential in plant and animal nutrition, and are the visual indicators of ripening for many fruits, including tomato. Whereas carotenoid synthesis is well characterized, factors regulating flux through the pathway are poorly understood at the molecular level. To exploit the impact of tomato genetic diversity on carotenoids, *Solanum pennellii* introgression lines were used as a source of defined natural variation and as a resource for the identification of candidate regulatory genes. Ripe fruits were analyzed for numerous fruit metabolites and transcriptome profiles generated using a 12 000 unigene oligoarray. Correlation analysis between carotenoid content and gene expression profiles revealed 953 carotenoid-correlated genes. To narrow the pool, subnetwork analysis of carotenoid-correlated transcription revealed 38 candidates. One candidate for impact on *trans*-lycopene and β-carotene accumulation was functionally charaterized, *SlERF6*, revealing that it indeed influences carotenoid biosynthesis and additional ripening phenotypes. Reduced expression of *SlERF6* by RNAi enhanced both carotenoid and ethylene levels during fruit ripening, demonstrating an important role for *SlERF6* in ripening, integrating the ethylene and carotenoid synthesis pathways.**

Keywords: fruit ripening, carotenoids, ethylene, ERF transcription factor, transcriptome analysis.

INTRODUCTION

Solanum lycopersicum (tomato) is an important crop with substantial and growing economic and nutritional impact, and is the most studied model system for fleshy fruit biology. Fruit ripening is a genetically programmed process that is modified by both endogenous and exogenous signaling systems. Biochemical changes in color, texture, flavor, aroma and nutritional content render the ripe fruit attractive to seed-dispersing organisms (Seymour *et al.*, 1993; Goff and Klee, 2006). Specific factors influencing ripening include developmental signals, hormones, light, temperature and nutrient status. The role of the plant hormone ethylene and a number of recently described ripening transcription factors have been well characterized at the molecular level (Giovannoni, 2004; Matas *et al.*, 2009; Klee and Giovannoni, 2011). The coloration of fleshy fruit is an indicator of ripening and an attractant for seed-dispersing organisms, and is tightly controlled by ripening regulators. Carotenoids, especially β-carotene and lycopene, are the principal ripe fruit pigments of tomato, and gene expression for the rate-limiting step in the carotenoid pathway, i.e. phytoene synthase (PSY), is regulated by ethylene (Maunders *et al.*, 1987).

Beyond their role in fleshy fruit coloration, carotenoids are indispensible in plant biology and human/animal diets as

essential components of photosynthesis and nutrition, respectively. Tomato fruit are a major source of lycopene and provitamin-A β-carotene in the human diet (Fraser and Bramley, 2004). Important derivatives of the carotenoid pathway in plants include the apocarotenoid plant hormone abscisic acid (Schwartz *et al.*, 2003), a number of flavor and aroma compounds (Goff and Klee, 2006), and strigolactones that serve as shoot branch-promoting hormones (Dun *et al.*, 2009). Although the catalytic steps of the carotenoid biosynthetic pathway have been described (Hirschberg, 2001; Fraser and Bramley, 2004; DellaPenna and Pogson, 2006; Cazzonelli and Pogson, 2010), the regulatory mechanisms that control carotenoid accumulation remain poorly understood. Carotenoid accumulation is spatially and temporally regulated in chloroplasts, and in the carotenoid-accumulating plastids, i.e. chromoplasts, of fruits and flowers. Changes in carotenoid accumulation have been demonstrated to correspond with changes in expression of genes encoding pathway enzymes (Fraser *et al.*, 1994; Ronen *et al.*, 2000; Bramley, 2002; Galpaz *et al.*, 2006). Several regulatory mechanisms have been suggested, including ethylene (Giovannoni, 2004), light (Mustilli *et al.*, 1999; Liu *et al.*, 2004), the availability of substrates produced through the methyl-D-erythritol-4-phosphate (MEP) pathway (Rodriguez-Concepcion *et al.*, 2001) and metabolic sequestration (Vishnevetsky *et al.*, 1999; Lu *et al.*, 2006). In addition, transcription factors have been found to control carotenoid levels (Toledo-Ortiz *et al.*, 2010). A number of transcription factors impacting ripening and thus carotenoid accumulation have been identified, including *RIN-MADS* (Vrebalov *et al.*, 2002), *CNR SQUAMOSA* promoter binding protein (Manning *et al.*, 2006), *TAGL1* MADS box (Vrebalov *et al.*, 2009), *LeHB-1* HBzip (Lin *et al.*, 2008) and *SlAP2a*, an AP2 gene (Chung *et al.*, 2010; Karlova *et al.*, 2011), although all have broad ripening effects and none specifically regulate a single fruit metabolic pathway.

More specific carotenoid regulators have been identified for non-fruit tissues. In Arabidopsis, modulating mRNA levels of the ethylene response factor (ERF) RAP2.2, which is capable of binding the *PSY* promoter, resulted in small carotenoid alterations in root calli (Welsch *et al.*, 2007). Phytochrome interacting factor 1 (PIF1), a basic helix–loop–helix (bHLH) gene family member, negatively regulates carotenoid accumulation by specifically binding the promoter of and repressing (*PSY*) *in planta* (Toledo-Ortiz *et al.*, 2010). Chromatin-modifying histone methyltransferase enzyme (SET DOMAIN GROUP 8, SDG8) was shown to be required for carotenoid isomerase (*CRTISO*) expression (Cazzonelli *et al.*, 2009). The absence of SDG8 alters the methylation of chromatin associated with *CRTISO*, thereby reducing gene expression, impairing lutein biosynthesis and increasing shoot branching.

Natural genetic variation has been exploited to overcome genetic bottlenecks during domestication, and to improve yield and quality (Tanksley and McCouch, 1997; Fernie *et al.*, 2006). Natural genetic variation also provides opportunities to unveil complex metabolic networks (Chan *et al.*, 2010; Sulpice *et al.*, 2010). Wild species such as *Solanum pennellii*, when compared with their domesticated relatives (*S. lycopersicum*), have allelic diversity, facilitating systems-level studies of gene expression and metabolic networks. Natural genetic diversity studies can be facilitated by the use of wild species introgression lines (ILs), recombinant inbred lines (RILs) or near isogenic lines (NILs), with defined and discrete fragments of the wild genome in the context of the cultivated genome (Zamir, 2001).

Genomics and/or systems approaches integrating transcript profiling with metabolite measurements have facilitated the study of the genetic mechanisms underlying metabolite variation. These approaches can help identify uncharacterized networks or pathways, in addition to candidate regulators of such pathways (Saito and Matsuda, 2010). In tomato, metabolomic data from isogenic, wild species and mapped ILs provide a powerful resource for identifying candidate genes regulating complex biochemical traits (Schauer *et al.*, 2006).

Numerous quantitative trait loci (QTL) influencing biological phenomena have been detected through genetic mapping, and a number have been isolated through candidate gene mapping and positional cloning (Salvi and Tuberosa, 2005). Subsequent gene isolations have typically resulted from highly targeted strategies through combined transcriptome/metabolome analyses using genetically well-characterized germplasm, such as mapped ILs. In this study, we applied ripe fruit transcriptional and metabolic profiling to a population of mapped single subchromosomal introgressions of *S. pennellii*. We mined candidate genes based on correlation analyses and identified a potential effector of carotenoid gene expression: *SlERF6*. This member of the ERF transcription factor family regulates fruit carotenoid accumulation. The results demonstrate the utility of systems-based analysis to identify genes controlling complex biochemical traits in crop species.

RESULTS

Transcriptome–carotenoid correlation

To assess relationships between fruit metabolites and gene expression, we exploited variation in carotenoid accumulation and gene expression in *S. pennellii* ILs (Eshed and Zamir, 1995; Pan *et al.*, 2000) (Figure 1a). Carotenoid quantitation and transcript profiling of ripe fruit pericarp tissues were performed by high-performance liquid chromatography (HPLC) and TOM2 (12K) oligonucleotide array analyses, respectively. We selected for comparative metabolite/transcriptome analysis from 23 ILs showing the greatest variation in concentrations of specific carotenoids

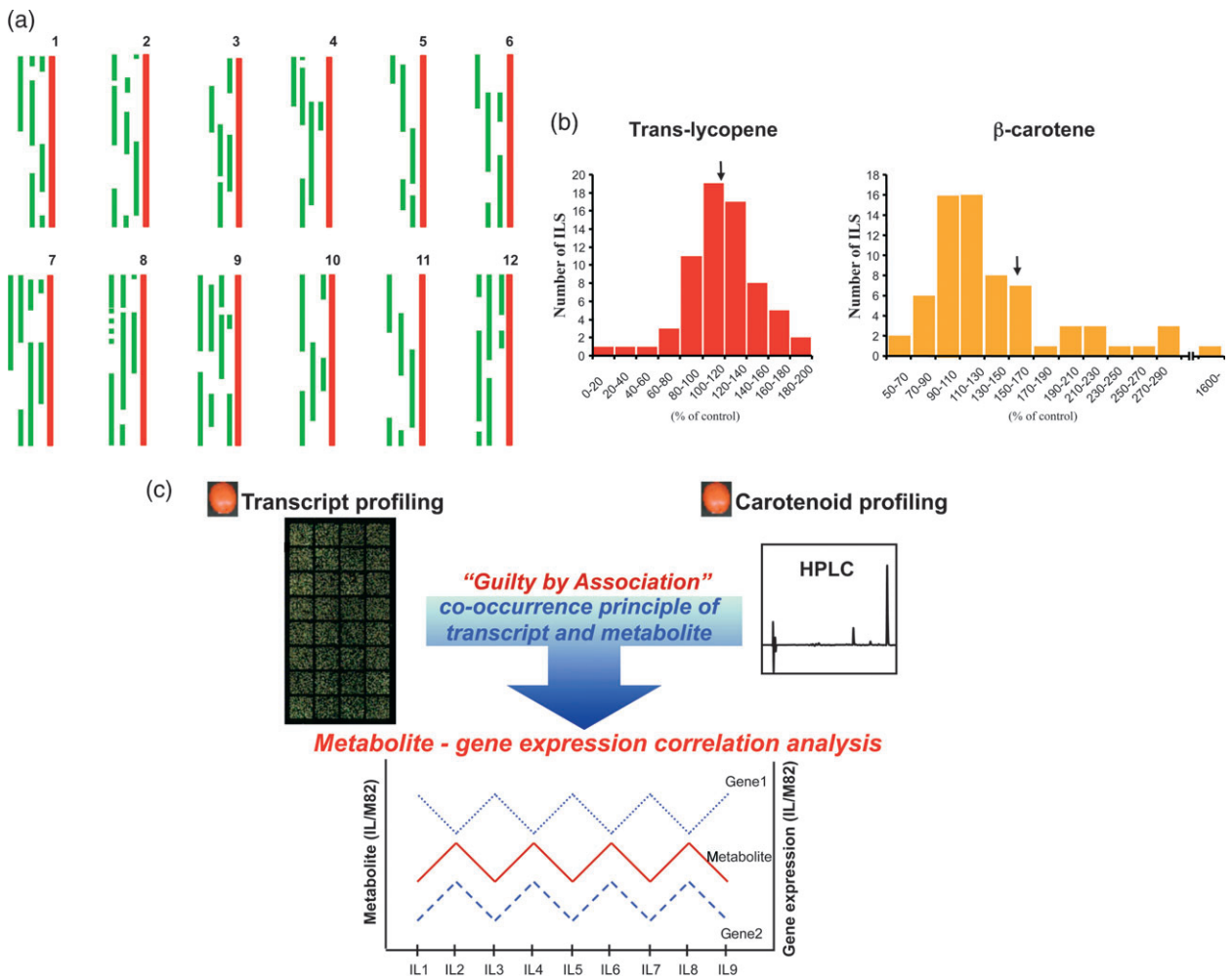

Figure 1. *Solanum pennellii* introgression lines (ILs) and variation of carotenoids in ripe fruit.
(a) The *S. pennellii* IL collection is composed of 76 lines, the overlapping segments of which can be defined as 107 bins. The 12 chromosomes of tomato are drawn as red bars and the *S. pennellii* introgressed segments are shown as green bars to the left of the chromosomes.
(b) Frequency distribution for each carotenoid measured in the *S. pennellii* ILs. The percentage value on the *x*-axis represents the value of the control (*S. lycopersicum* backcross parent M82), and the population means are indicated by black arrows.
(c) Experimental concept for integrating transcriptome and carotenoid profiles by correlation analysis. Direct comparison of each IL with the wild-type control 'M82' was employed by microarray analysis using the TOM2 oligonucleotide array (12K) (Enfissi *et al.*, 2010) (http://ted.bti.cornell.edu). Transcriptome analysis was conducted using between six and eight independent microarrays and a dye-swap design. Carotenoids from the same ripe fruit tissues used for transcriptome analysis were also quantified for carotenoids using HPLC. Ripe fruit tissues were pooled from a minimum of 20 fruits from the wild type and each of the ILs, and the samples were split into four subpools, and then used in microarray hybridization and carotenoid quantification. Comparisons of transcriptome and metabolite data were based on the co-occurrence principle between metabolite and gene expression level (Saito and Matsuda, 2010). Candidate genes positively or negatively correlated with each carotenoid were identified (Table S1).

(especially *trans*-lycopene and β-carotene, which are the major tomato carotenoids) (Figure 1b). We created a correlation matrix of all pairwise comparisons among carotenoids, and between carotenoid concentrations and gene expression. Each carotenoid showed wide ranges of concentrations in the pericarp tissues derived from these lines, indicating that the ILs contain significant carotenoid variation (Figure 1a,b).

A total of 953 unigenes could be correlated with one or more of the nine measured carotenoids, as defined by a correlation coefficient $|r| > 0.5$ ($P < 0.01$) for at least one carotenoid, based on the co-occurrence between metabolite and mRNA levels (Figure 1c). The full set of correlation coefficients (Table S1) is presented as a heat map in Figure 2a.

We also detected significant correlations among the various carotenoids analyzed in the IL fruit. Several carotenoid correlation modules that may be indicative of regulators or regulatory networks were found. For example, phytoene, phytofluene, *cis*-lycopene and *trans*-lycopene are acyclic, and all resided in one correlation module, whereas δ-carotene and lutein were members of another correlation module (Figure 2b). Furthermore, correlation patterns between transcripts and carotenoids were similar

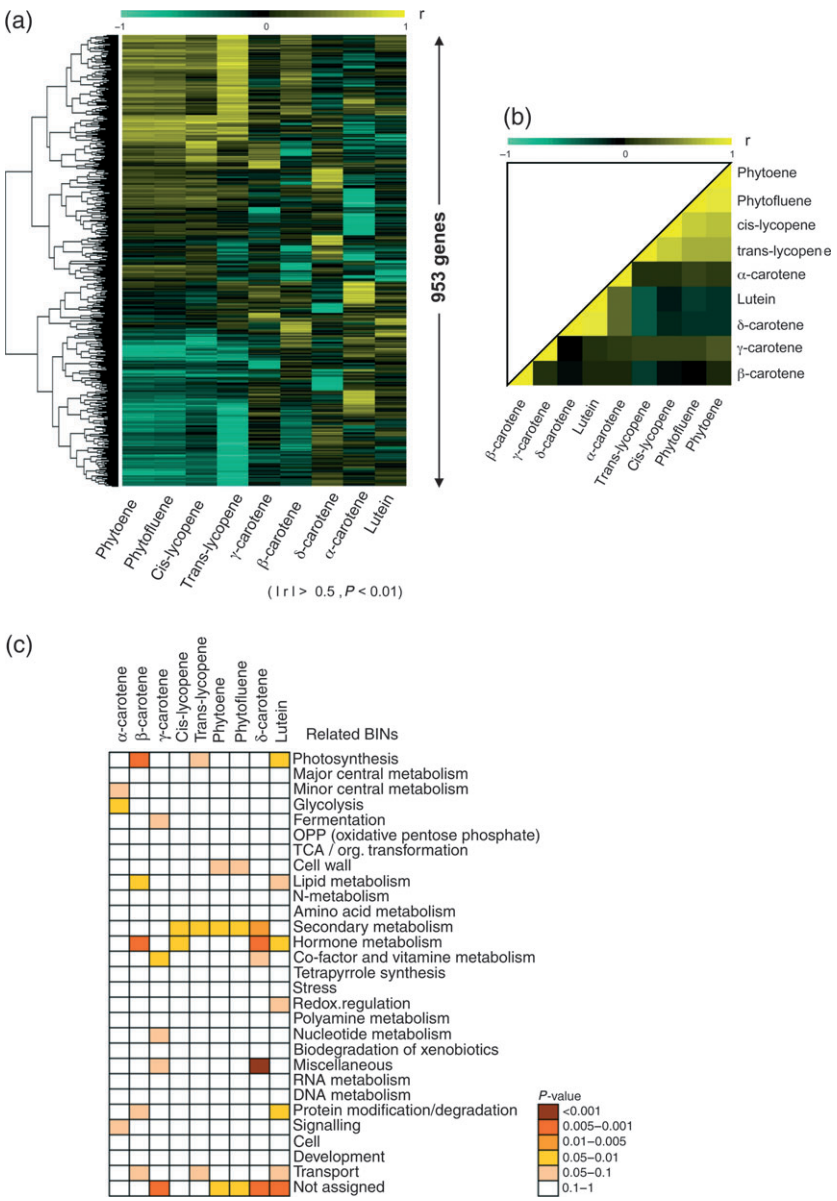

Figure 2. Heat map of gene expression–carotenoid correlation. Correlation coefficients and significance were calculated by the Pearson algorithm using R.
(a) Out of 12 000 unigenes, 953 genes were significantly correlated with carotenoids ($|r| > 0.5$; $P < 0.01$). The rows in the heat map are the 953 genes clustered by their expression patterns and the columns are carotenoid metabolites.
(b) A heat map of carotenoid–carotenoid correlation. The intensity of cyan and yellow colors in the heat map indicates the level of positive and negative correlation, respectively.
(c) A heat map of over-represented categories from the correlation data. The data was subjected to a Wilcoxon test and the *P* values were displayed. Colored areas are significantly enriched in each carotenoid relative to other functional classes.

among the four acyclic carotenoids, although the pattern for δ-carotene was distinct from the pattern of lutein (Figure 2a). These results suggest that the regulatory control of accumulation of the acyclic carotenoids is likely similar, whereas lutein and δ-carotene are probably influenced by different regulators.

In order to categorize carotenoid-related activities, we conducted MapMan analysis (Usadel *et al.*, 2005) to visualize over-represented functional categories derived from correlation data (Figure 2c). For instance, a functional class related with secondary metabolism was over-represented for all acyclic carotenoids and δ-carotene, suggesting relevance to carotenoid biosynthesis as significant numbers of candidate genes in this functional class were isoprenoid or carotenoid biosynthetic genes. A photosynthesis-related BIN was over-represented in association with β-carotene and lutein, thereby supporting their well-documented roles in light harvesting and photoprotection (Lichtenthaler, 2007). Among the carotenoid-correlated genes, several were previously reported to function as tomato fruit carotenoid or color regulators (Table S2). Examples include the ethylene receptor homologs, *Never ripe* (*Nr*) ($r = -0.66$) and *LeETR4* (Tieman *et al.*, 2000) ($r = -0.55$), which displayed strong negative correlation with *trans*-lycopene. Carotenoid or isoprenoid biosynthetic pathway genes, 1-deoxy-D-xylulose-5-phosphate synthase (*DXS*) ($r = 0.58$ for *cis*-lycopene;

Lois *et al.*, 2000), chromoplast lycopene β-cyclase (*CYC-B*) (r = 0.6 for δ-carotene; r = 0.34 for β-carotene; Ronen *et al.*, 2000), chloroplast lycopene β-cyclase (*LCY-b*) (r = 0.49 for β-carotene; Pecker *et al.*, 1996), lycopene ε-cyclase (*LCY-ε*; r = −0.73 for *trans*-lycopene; Ronen *et al.*, 1999) and β-ring carotene hydroxylase (*CrtR-b2*) (r = 0.54, γ-carotene; Galpaz *et al.*, 2006) also displayed correlation with carotenoids, as might be anticipated logically. Indeed our data indicate that gene expression of the pathway enzymes was positively correlated with the corresponding catalytic product and negatively correlated with their upstream substrate. The fact that a number of genes previously associated with tomato ripe fruit carotenoid content were recovered indicates the analysis is robust and genes identified via this process are worthy of validation.

Targeted gene–carotenoid network analysis reveals putative regulators of carotenoid accumulation

To unveil regulatory genes underlying carotenoid accumulation in tomato fruit, we used correlation network analysis to identify the transcription factors that most strongly associate with carotenoid levels (Figure 3). We first analyzed the available digital expression data (Fei *et al.*, 2004) to define abundant mRNAs corresponding to putative transcription factors (TFs) in fruit. Digital expression levels were assigned to three classes (low, intermediate and high). Thirty-eight putative TFs (Table S3) expressed in fruit, as determined by digital expression analysis, were employed for correlation network analysis with nine carotenoids using a multinetwork tool (Shannon *et al.*, 2003) (Figure 3).

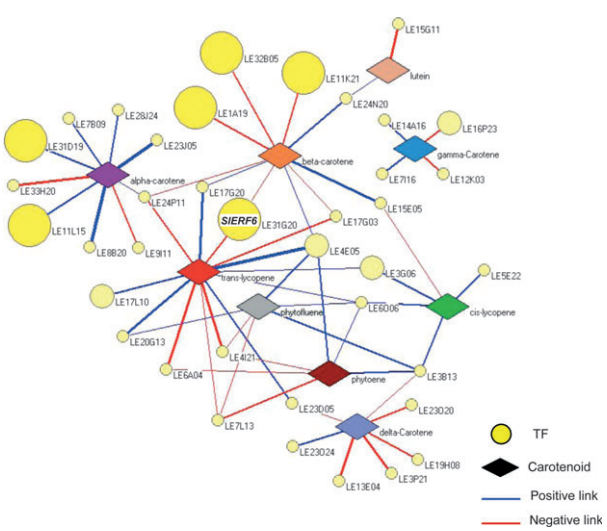

Figure 3. Carotenoid-associated transcription factor (TF) correlation network of ripe tomato fruit. TFs were selected from Table S1 and their respective correlations (P < 0.05) are indicated. Cytoscape (Cline *et al.*, 2007) was used to visualize the resulting subnetwork. TFs (nodes) are drawn as yellow circles and carotenoids are drawn as diamonds. The sizes of the circles represent transcription amplitude (high, intermediate and low) in fruit, based on the digital expression pattern of tomato expressed sequence tags (Fei *et al.*, 2004). Blue and red lines indicate positive and negative carotenoid correlations, respectively. The thickness of lines represents the level of correlation coefficience. Gene ID numbers from the TOM2 microarray are shown with nodes. *SlERF6* is highly expressed in tomato fruit, and the corresponding node is negatively linked with *trans*-lycopene and β-carotene, the two major carotenoids in ripe tomato fruit.

Detailed interpretation of the network helps define the hypothetical relationship among participants and a strategy to test said hypothesis. For example, if a candidate gene is highly expressed in fruit and inversely correlated with a given metabolite, a targeted knock-down approach may be appropriate to confirm the predicted function as well as demonstrate a metabolic engineering strategy for the elevation of said metabolite. For a candidate showing low expression in the context of available promoters and positive correlation, a transgenic overexpression strategy would be appropriate to both demonstrate positive action and elevate a compound of interest.

Six ripening-associated TFs (*RIN-MADS*, *CNR*, *LeEIL*, *Le-HB1*, *SlAP2a* and *TAGL1*) have been previously shown to be ripening regulators with associated fruit carotenoid phenotypes, via mutant or transgenic studies. Among the 38 TFs identified in this study with carotenoid association, *LeEIL* (LE28J24, r = 0.56) (Tieman *et al.*, 2001) and *RIN-MADS* (LE31D19, r = 0.57) were recovered. *Le-HB1* could not be identified as it is not included in the TOM2 array. *SlAP2a* (LE20P08) was correlated with γ-carotene under lower criteria (P = 0.03. r = −0.46). *TAGL1* and *CNR* did not show significant correlation in this analysis, indicating that there is no significant variation in activity or expression of the *S. pennellii* allele. We selected LE31G20, an uncharacterized member of the AP2-ERF transcription factor family, denoted as *SlERF6* for functional analysis, because: (i) it is highly expressed in ripening fruit; (ii) it has strong correlation values with ripe fruit carotenoids; and (iii) it lacked prior characterization in the context of fruit carotenoid content. Specifically, *SlERF6* is inversely correlated with *trans*-lycopene (r = −0.56) and β-carotene (r = −0.44).

SlERF6 expression is dependent upon both ethylene and non-ethylene control during fruit ripening

A full-length cDNA sequence of *SlERF6* (GenBank accession JN616265) was identified from a cDNA clone (cLEG51D19). This gene codes for a predicted polypeptide of 254 amino acids (Figure S1A). SlERF6 specifically belongs to group VII of the ERF family (Nakano *et al.*, 2006), as determined by phylogenic analysis with five Arabidopsis and four tomato ERF families (Figure S1B). Although multiple alignments were conducted using the same subfamily group (Figure S1A), sequences were highly variable except for the conserved ERF domain (highlighted by a red box in Figure S1A) suggesting that group-VII family members are likely to have distinct functions.

SlERF6 expression was monitored via quantitative RT-PCR in *S. lycopersicum* cv. Ailsa Craig (hereafter AC) fruits across a developmental continuum of fruit maturation (Figure 4a). *SlERF6* transcript levels increase at the mature green (MG) stage, reach a maximum level at 3 days post breaker (B + 3), and decrease through at least B + 7, thus showing correlation with later fruit development and ripening. Ethylene is a well-known ripening hormone also associated with carotenoid accumulation (Hirschberg, 2001; Giovannoni, 2004), and some AP2/ERF genes are known to be induced by ethylene (Gutterson and Reuber, 2004). Thus, the effect of exogenous ethylene relative to *SlERF6* mRNA accumulation was assessed in MG fruit. MG fruit exposed to ethylene for 16 h showed that *SlERF6* expression is responsive to exogenous ethylene (Figure 4b). The expression level of *SlERF6* in the wild type and in the ripening impaired mutants *rin* (*ripening inhibitor*), *nor* (*non-ripening*) and *Nr* (*Never-ripe*) was also investigated, and was reduced in all three low-ethylene and ripening-impaired mutants (Figure 4b). Whereas *SlERF6* transcript levels displayed twofold reductions in *Nr* and *rin* fruit, *SlERF6* in *nor* showed a fivefold reduction (Figure 4c). As *rin* and *nor* are non-climacteric fruits that fail to produce ripening ethylene, the reduced *SlERF6* expression is consistent with ethylene control. *SlERF6* expression was further assayed in ethylene-treated *rin* and *nor* fruit. Interestingly, whereas *SlERF6* was induced in ethylene-treated *rin* fruits to a level similar to ethylene-treated wild-type fruit, expression was not altered in ethylene-treated *nor* fruits (Figure 4d). These results imply that *SlERF6* is regulated by ethylene but also by a *NOR*-dependent pathway. Similar expression regulation has been observed for the tomato *E8* and *ACS2* genes (Yokotani *et al.*, 2004), suggesting a unique regulatory pathway operating during ripening that influences a specific subset of genes.

Reduced expression of *SlERF6* enhances carotenoid and ethylene levels during ripening, confirming the network association

To verify the correlation network analysis and predicted function of *SlERF6* as a negative regulator of carotenoid accumulation, we deployed an RNAi strategy to repress this gene. Gene-specific sequences (316 bp) at the 3′ region of *SlERF6* and excluding the conserved ERF domain were used for RNAi construct development in pHELLSGATE2 (Wesley *et al.*, 2001), and introduced to AC tomato via *Agrobacterium*-mediated transformation. Three independent transgenic lines (lines 4, 13 and 23; Figure S2), showing increased carotenoids and reduced *SlERF6* expression, were selected for further characterization. The *SlERF6* transcript was reduced to approximately 10% of control levels in ripening fruit, with the greatest effect at the early ripening or breaker (BR) stage, and with partial restoration of transcript levels by the red ripe B + 7 stage (Figure 5a). Carotenoids were extracted from pericarp tissues of transgenic and control fruits at different stages and quantified by HPLC. All three RNAi lines exhibited maturing fruit with approximately 1.5–2.5-fold increases in total carotenoids (Figure 5b; Table 1). Carotenoid levels were most different in the transgenic versus control fruit by B + 7, and persisted during later development. Carotenoid phenotypes were maintained and increased in the T_1 and T_2 generations, indicating the

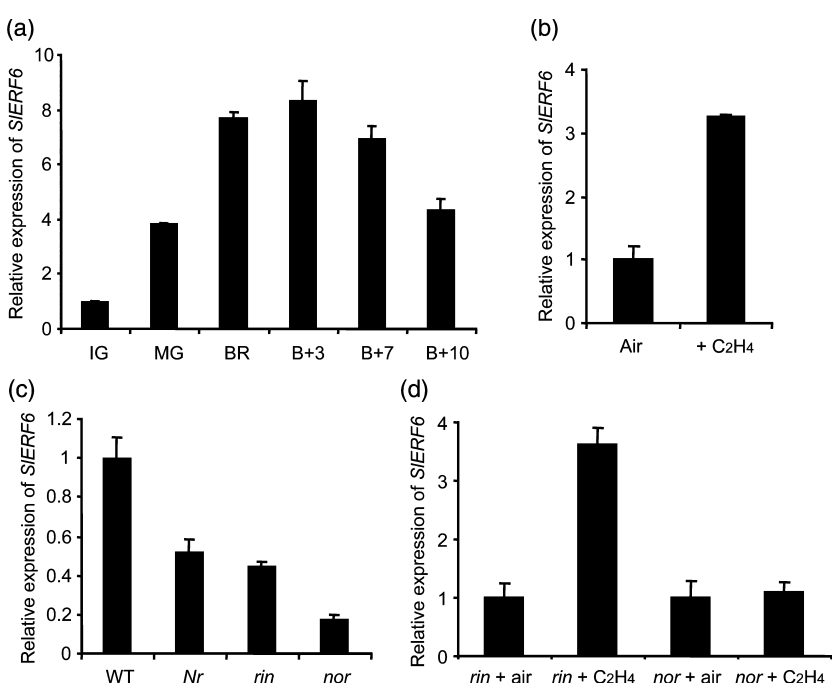

Figure 4. Gene expression of *SlERF6* in tomato fruit.
(a) *SlERF6* transcript levels at various fruit developmental stages. Transcript levels determined by quantitative RT-PCR are expressed relative to IG. IG, immature green; MG, mature green; BR, breaker; B + 3, 3 days post breaker; B + 7, 7 days post breaker; B + 10, 10 days post breaker.
(b) Ethylene responsiveness of *SlERF6*. RNA samples were extracted from MG fruit of cultivar AC treated for 16 h with air or with 20 μL L^{-1} ethylene.
(c) *SlERF6* transcript levels in *Nr*, *rin* and *nor* fruit (same age post-anthesis as B + 10 control fruit). *Nr*, *never-ripe*; *rin*, *ripening inhibitor*; *nor*, *non-ripening*. Results are means ± SEs from two measurements of three independent biological replicates.
(d) Ethylene responsiveness of *SlERF6* in ethylene-treated *rin* and *nor* fruits.

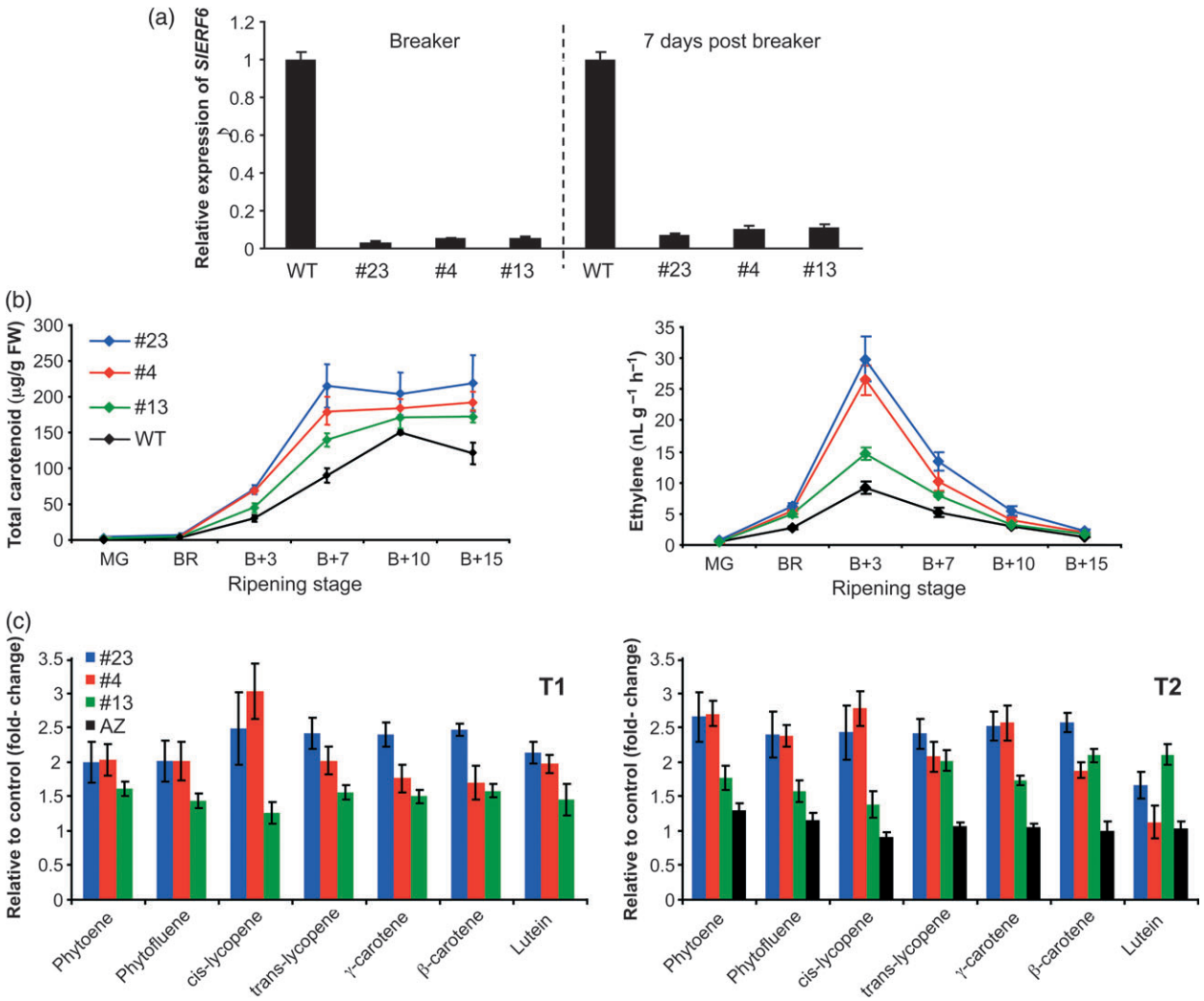

Figure 5. Elevated carotenoids and ethylene in ripe *SlERF6*-RNAi fruit.
(a) Transcript levels of *SlERF6* in wild-type and T_2 RNAi-transgenic tomatoes of breaker (BR) and fruits at 7 days post breaker (B + 7). Transcript levels relative to control (azygous segregant line) are presented.
(b) Carotenoid and ethylene level in wild type and three independent *SlERF6*-RNAi lines during ripening. Total carotenoids were extracted from pericarp tissues of T_1 lines ($n > 5$) with AC, and quantified by HPLC. Ethylene was detected by GC ($n > 10$).
(c) Stability of carotenoid profiles in T_1 and T_2 generations of *SlERF6* RNAi lines. HPLC analysis of carotenoid accumulation in B + 7 fruits of *SlERF6*-RNAi lines presented as a percentage of control (cv. AC). Standard errors are indicated for a minimum of five fruits per sample; AZ, azygous line.

Table 1 Carotenoid content of *SlERF6*-RNAi transgenic tomatoes (T_1) in ripe fruit (B + 7)

	Carotenoid content (mg per g fresh weight)			
Carotenoid	WT	RNAi line 23	RNAi line 4	RNAi line 13
Phytoene	1.85 ± 0.36	3.70 ± 0.55 (1.99)	3.76 ± 0.41 (2.03)	2.98 ± 0.19 (1.61)
Phytofluene	1.27 ± 0.22	2.56 ± 0.38 (2.01)	2.57 ± 0.35 (2.01)	1.84 ± 0.13 (1.44)
cis-lycopene	0.97 ± 0.23	2.42 ± 0.51 (2.48)	2.95 ± 0.49 (3.03)	1.23 ± 0.16 (1.26)
trans-lycopene	75.70 ± 7.41	182.16 ± 17.32 (2.41)	152.7 ± 15.8 (2.02)	117.98 ± 7.7 (1.56)
γ-carotene	2.13 ± 0.21	5.07 ± 0.373 (2.39)	3.73 ± 0.42 (1.76)	3.19 ± 0.21 (1.50)
β-carotene	7.22 ± 1.29	17.81 ± 0.65 (2.47)	12.27 ± 1.73 (1.70)	11.44 ± 0.68 (1.58)
Lutein	0.65 ± 0.13	1.40 ± 0.10 (2.14)	1.29 ± 0.09 (1.97)	0.95 ± 0.15 (1.45)
Total carotenoid	89.81 ± 9.84	215.13 ± 19.88 (2.39)	179.29 ± 19.29 (2.00)	139.6 ± 9.21 (1.55)

Fold changes compared with the wild type.

stability of transgene effects (Figure 5c). *SlERF6* effects could be seen on all carotenoids as an approximately twofold elevation compared with the control. We thus hypothesized that *SlERF6* may exert its influence at a point early in the carotenoid biosynthesis pathway. Ethylene has been shown to be an important component of ripening carotenoid accumulation. We thus analyzed ethylene evolution from *SlERF6*-repressed fruit, and ripening fruits from all three transgenic lines consistently produced ethylene at higher rates than fruits from the azygous control fruits at all of the ripening stages monitored (Figure 5b). Transgenic lines 23 and 4 produced more ethylene than line 13, and displayed a similar elevation of carotenoid levels. It is interesting that *SlERF6* is responsive to ethylene (Figure 4b), suggesting that *SlERF6* may be a component of a feedback restriction in ethylene production during ripening, similar to *SlAP2a* (Chung *et al.*, 2010). In contrast to *SlAP2a*-repressed lines that produce elevated ethylene and ripen approximately 1 week earlier than normal (also in AC), the time to the onset of ripening in *SlERF6*-repressed fruit was not altered (Table S4).

To identify genes influenced by *SlERF6*, we conducted fruit transcriptome analysis using the TOM2 microarray (Table S5). We identified differentially expressed genes between BR fruits of azygous and RNAi lines (pooled samples of lines 4 and 23). Most of the genes identified (85 of 99) showed upregulation (by more than twofold) in transgenic lines versus azygous BR fruit. Among differentially expressed genes, a number including *HSP21* (Neta-Sharir *et al.*, 2005), *ACS2* (Barry *et al.*, 2000) and *ACO1* (Barry *et al.*, 1996) were upregulated, consistent with their roles in ethylene and carotenoid accumulation. We validated these results and those of additional genes by quantitative RT-PCR from BR and B + 7 fruits (Figure 5a) in all three repressed lines and controls. Specifically, *HSP21* and *DXS* expression levels were significantly increased in the BR and B + 7 stages of all three RNAi lines. *ACO1* and *ACS2* were upregulated in BR, whereas *ACO3* was upregulated in the BR and B + 7 stages, suggesting that *SlERF6* negatively regulates *HSP21* and *DXS* to control carotenoid levels, and *ACO1*, *ACO3* and *ACS2* for ethylene (which in turn may impact on carotenoid levels as well). Other carotenoid and ethylene-related genes were not substantially altered (including *PSY1*), suggesting that the overproduction of ethylene and carotenoids is probably mediated primarily via the previously mentioned genes. Nevertheless, we cannot rule out the possibility that *SlERF6* plays a primary role in regulating ethylene synthesis, which in turn is primarily responsible for changes in other fruit traits, including carotenoid synthesis. It has been reported that the overexpression of *HSP21* resulted in increased carotenoids during ripening (Neta-Sharir *et al.*, 2005), and that fruit-specific over-expression of *DXS* increases carotenoid levels by 1.6-fold (Enfissi *et al.*, 2005), which is consistent with our observations.

Although *SlERF6* repression clearly impacts on the accumulation of *HSP21*, *DXS*, *ACO1*, *ACO3* and *ACS2* mRNAs, it is not clear whether these changes reflect activities resulting from elevated ethylene (i.e. an indirect effect of *SlERF6* repression) or more direct interactions. We investigated the accumulation of these mRNAs in response to a 16-h exogenous 20 μL L^{-1} ethylene treatment of wild-type MG (pre-climacteric) fruit. mRNAs for the *E4* and *E8* genes were also assayed as positive controls of exogenous ethylene treatment (Lincoln *et al.*, 1987). Figure 6b shows that levels of *ACO1*, *ACO3* and *ACS2* in ethylene-treated unripe wild-type fruit increased significantly, whereas *DXS* increased to a lesser extent, although significantly (by $\sim$1.8-fold). In contrast, *HSP21* accumulation was not affected by ethylene treatment, although the level of this transcript increases during ripening (Neta-Sharir *et al.*, 2005), consistent with a negative regulatory impact of *SlERF6*. *ACO1*, *ACO3* and *ACS2* encode the final steps in ethylene synthesis, and are themselves induced by ethylene during ripening (Barry *et al.*, 1996, 2000), complicating the interpretation of this experiment. However, the lack of *HSP21* expression in ethylene-treated fruit suggests that targets of *SlERF6* extend beyond genes in the ethylene biosynthesis pathway, and may reflect more specific targets for carotenoid pathway control beyond pleiotropic ethylene effects. We note that no other obvious phenotypes were observed in non-fruit tissues, and no obvious changes in fruit size or weight were observed.

DISCUSSION

A systems strategy reveals putative regulators of carotenoid accumulation

We employed a systems approach to identify regulatory genes that impact on carotenoid accumulation, as the regulation of this aspect of ripening and fruit nutritional quality remains poorly understood, and most fruit pigmentation mutants characterized to date are the consequence of defects in carotenoid biosynthesis (Hirschberg, 2001; Fraser and Bramley, 2004). We further demonstrate that coupling metabolite variation with transcriptome changes caused by natural genetic variation can be used as a general method for identifying metabolite/gene network associations lacking additional information regarding the specific nature of candidate regulators. The approach requires a well-characterized population as a source of genetic variation, such as ILs or RILs. Here, we employed previously mapped *S. pennellii* ILs (Eshed and Zamir, 1995; Pan *et al.*, 2000), one of the more divergent wild species from the cultivated tomato (Moyle, 2008). These lines harbor allelic diversity, impacting on the chemical composition of numerous metabolites (Lippman *et al.*, 2007), including carotenoids, as shown here

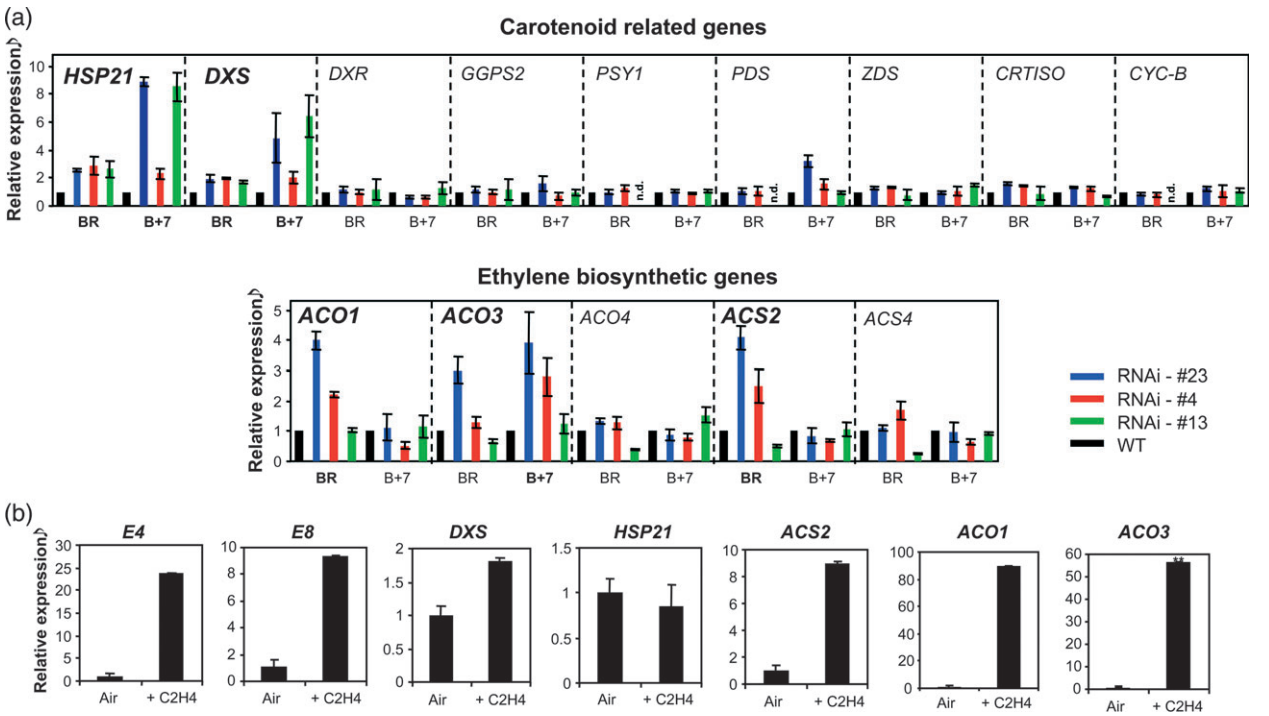

Figure 6. Confirmation of gene expression patterns influenced by *SlERF6* repression.
(a) QRT-PCR analysis of carotenoid and ethylene-related genes in *SlERF6*-RNAi fruit. Analysis of transcript levels of carotenoid and ethylene metabolism related genes by quantitative RT-PCR. Genes and their primers are listed in Table S5. Transcript levels relative to the wild type are presented.
(b) Ethylene responsiveness of carotenoid and ethylene-related genes in ethylene-treated MG fruits. All genes are significantly responsive to ethylene ($P < 0.01$) except *HSP21* ($P = 0.1583$), by Student's *t*-test. The data represent the mean values (±SEs) of duplicate experiments from three independent biological samples; n.d., not determined.

(Figure 1b) and by others (Liu *et al.*, 2003). Correlation analysis of fruit metabolome and transcriptome data resulted in multiple new hypotheses regarding regulatory relationships between carotenoids and both characterized and numerous previously uncharacterized transcription factors (Figure 1c). A similar strategy could be undertaken for any of a number of metabolite–gene associations, and indeed hundreds of metabolites, volatiles and additional traits from these ILs can be accessed online, along with the complete expression data set generated here for correlation analysis (Fei *et al.*, 2011). We note that many of the metabolites measured and available on this database were generated from fruit of the same trial from which the transcriptome data described here were generated.

Structurally similar metabolites derived from the same or related pathways often show correlations reflecting precursor–product or common regulatory relationships (Saito and Matsuda, 2010). For example, concentrations of acyclic carotenoids are often highly correlated with each other, and their correlations with specific gene mRNA level changes are similar, implying that common regulators reside in such metabolite/enzyme/genetic regulator modules.

Recovery in our correlation analyses of known mediators (*Nr*, *LeETR4*, *CYC-B*, *LCY-E*, *CRTR-B2*, *RIN* and *LeEIL*; Table S2) of carotenoid accumulation demonstrate the predictive power of this analysis. However, interestingly we did not recover *PSY1*, a well-documented regulator of carotenoid accumulation, as a rate-limiting step in the pathway. Some genes may demonstrate correlations with metabolites only under particular conditions or at specific stages of development (Saito *et al.*, 2008). As we only analyzed fully ripe fruit, we may have missed important transition stages where known carotenoid influencing genes such as *CNR* and *TAGL1* have optimal influence. Correlations between transcript levels of several isoprenoid/carotenoid biosynthetic genes and carotenoid content in maize kernels was highly dependent on developmental timing, in that a clear correlation of *PSY1* and endosperm carotenoid content was observed at 20 but not 25 days after pollination (Vallabhaneni and Wurtzel, 2009).

A positive correlation between *CYC-B* and carotenoid (γ-carotene and β-carotene) levels was observed. Therefore, β-carotene formation from lycopene appears to be principally under transcriptional regulation (Hirschberg, 2001; Fraser *et al.*, 2007). By contrast, the lack of correlation between *PDS*, *ZDS* and *CRTISO* expression and carotenoid levels in this study, and reported previously (Fraser *et al.*, 2007), suggests that post-transcriptional regulation may

operate in these cases. Also, although we focus on metabolites regulated by gene expression changes because these are the parameters we measure, we cannot exclude other modes of regulation. Indeed carotenoid catabolites, including ABA and strigolactones, are important plant hormones that have multiple functions, and could be added to this analysis when measured.

By emphasizing transcription factors, we generated a TF–carotenoid subnetwork (Figure 3) to identify candidate regulators impacting on carotenoid accumulation. Information on the spatiotemporal pattern of gene expression/metabolite accumulation can facilitate gene function and hypothesis generation. Thus, predictions for candidate transcription factors deduced from the correlation data were reinforced by in-house digital expression data (Fei *et al.*, 2004). Of course, at this level of analysis we cannot distinguish between primary or direct effectors of carotenoid synthesis via interaction with biosynthetic genes and those who exert their impact indirectly through intermediaries. We have identified 38 TFs that are expressed in fruit and significantly correlated with carotenoids. To validate the subnetwork analysis, we selected *SlERF6*, a putative negative regulator of the two major fruit carotenoids. This gene is highly expressed in fruit and transcriptionally regulated by ripening (Figure 4a), and resulted in altered carotenoid levels when repressed, consistent with its hypothesized negative regulatory role.

Validation of a network prediction reveals the regulatory role of *SlERF6* in fruit ripening

Thirty-eight tomato transcription factors (Table S3) showed gene expression patterns that suggested participation in the regulatory network influencing fruit carotenoid profiles and content (Figure 3). Several of the identified transcription factors were previously shown to influence fruit carotenoids (Table S2). To validate the predicted carotenoid network, we selected one of the best-supported nodes for which functional data was not available: *SlERF6*. RNAi suppression of *SlERF6* increased carotenoid and ethylene accumulation during ripening (Figure 5). In three independent RNAi lines, the mRNA levels of *DXS*, *HSP21*, *ACS2*, *ACO1* and *ACO3* were increased in one or more ripening stages (Figure 6a). Isoprenoids, including carotenoids, are formed via the MEP pathway (Eisenreich *et al.*, 2001). From pyruvate and D-glyceraldehyde-3-phosphate, 1-deoxy-D-xylulose-5-phosphate (DXP) synthesis is catalyzed by DXP synthase (DXS) (Lois *et al.*, 1998). The formation of MEP is catalyzed by DXP reductoisomerase (DXR). A subsequent series of head-to-tail condensations results in the formation of geranylgeranyl diphosphate (GGPP), the immediate precursor of carotenoids. *DXS* mRNA accumulation in tomato shows developmental and organ-specific accumulation, and a strong correlation with carotenoid synthesis. DXS may be the first regulated step in carotenoid biosynthesis during early fruit ripening (Lois *et al.*, 2000), supported by the fact that fruit-specific overexpression of *DXS* increased the carotenoid content (Enfissi *et al.*, 2005). Overexpression of the chloroplast-targeted small heat-shock protein HSP21 was also shown to promote carotenoid accumulation during tomato fruit maturation (Neta-Sharir *et al.*, 2005). The fact that *DXS* and *HSP21* are substantially altered in expression by the repression of *SlERF6* indicates that the impact of this gene on fruit carotenoid content may be exerted at least in part through the regulation of DXS and HSP21 gene expression.

ACS2 and *ACO1* encode the major enzymes responsible for ethylene biosynthesis in tomato fruit ripening (Barry *et al.*, 1996, 2000). *ACO3* mRNA is predominantly expressed in flowers, and is slightly induced during ripening and leaf senescence (Barry *et al.*,1996). *SlERF6* suppression resulted in the elevated expression of all three genes in ripening fruit, suggesting a regulatory function in ethylene synthesis through the genes encoding the final steps of the pathway. Whether or not SlERF6 is a direct regulator of these genes or an indirect regulator of carotenoid accumulation, for example as shown through manipulation of fruit polyamine levels (Mehta *et al.*, 2002), remains uncertain.

ACS and *DXS* expression are regulated by ethylene (Figure 6), whereas *HSP21* is not, suggesting that some genes affected by SlERF6 are acting downstream of SlERF6-mediated ethylene synthesis, whereas others are influenced by more direct means. ERF gene family members are known to bind the the target DNA GCC box via the highly conserved ERF domain (Nakano *et al.*, 2006). GCC and GCC-like elements in promoter regions of *HSP21*and *ACO1* could be identified in the tomato genome sequence (http://solgenomics.net). A GCC box (GCCGCC) element in the *ACO3* promoter and a GCC box-like [T(C)CCGCC] element associated with *ACS2* have been previously reported (Zhang *et al.*, 2009).

The majority of genes substantially influenced by *SlERF6* repression were upregulated, indicating that the primary function of SlERF6 is via negative regulation (Figure 6; Table S5). In this context there are several possibilities as to the specific activity of SlERF6, including: (i) transcriptional repressor; (ii) transcriptional activator of a transcriptional repressor; and (iii) recruiter of transcriptional repressor. It is noteworthy that class-II ERF subfamily members have been shown to contain a conserved repressor domain, termed the EAR motif, in their C-terminal region (Ikeda and Ohme-Takagi, 2009). However, SlERF6 belongs to the group-VII ERF subfamily (Figure S1). Multiple alignments of tomato and Arabidopsis group-VII family members showed divergent variation of sequences beyond the conserved ERF domain, suggesting that this family may have diverse functions and modes of action. Arabidopsis RAP2.2 is a member of this family, is involved in stress responses (Nakano *et al.*, 2006; Hinz *et al.*, 2010) and binds to the *PSY* promoter *in vitro*, consistent with a role in carotenoid regulation. However, modulating transcript levels of RAP2.2 resulted in negligible

carotenoid alterations in root calli (Welsch *et al.*, 2007). No significant carotenoid and chlorophyll variation in leaves was observed in the *SlERF6*-RNAi lines (data not shown), suggesting that the functions of these genes may be under tissue-specific constraints.

Results from the transgenic repression of *SlERF6* suggest it exerts negative feedback control over ethylene production during ripening. SUBMERGENCE 1A (SUB1A) is a rice-VII ERF, and participates in a feedback restriction mechanism for ethylene biosynthesis during submergence (Fukao *et al.*, 2006), suggesting a conserved negative regulatory role in ethylene biosynthesis for the group-VII ERF family. Interestingly, *SlERF6* is induced by ethylene in maturing fruit (Figure 4b), suggesting that its own expression is connected into the mechanism of fruit ethylene homeostasis to insure balanced ethylene synthesis during ripening. The modulation of ethylene synthesis may be necessary to insure that limited ethylene receptors do not become saturated, and thereby maintain the capacity to respond (O'Malley *et al.*, 2005). It is also noteworthy that carotenoid synthesis genes influencing final carotenoid profiles, such as *PSY1* and *CYC-B*, are regulated by ethylene (Alba *et al.*, 2005), and thus a mechanism for ethylene control may be necessary to achieve optimal fruit pigmentation. The molecular mechanism defining exactly how *SlERF6* and the *SlAP2a* negative regulators of ethylene synthesis and ripening exert their effects (for example as primary or secondary regulators of ethylene synthesis genes), or possibly interact during fruit maturation, remains to be determined.

The present approach of integrated transcriptome and metabolite profiling using ILs as a source of variation of gene expression and metabolites provided insight into multiple previously uncharacterized candidate carotenoid regulators. SlERF6 may integrate ethylene-dependent and -independent (e.g. NOR-dependent) regulatory activities to allow for the fine-tuning of signal outputs. The increased ease and efficiency of RNA-seq and metabolomics tools will facilitate future systems analyses and provide more insight into the nature of transcript–metabolite networks impacted by the genetic diversity underlying fruit ripening.

EXPERIMENTAL PROCEDURES

Plant material

Solanum pennellii ILs (Eshed and Zamir, 1995; Pan *et al.*, 2000) and the *S. lycopersicum* control M82 were grown in Florida, spring 2004, as described by Tieman *et al.* (2006). Transgenic and control plants were grown in glasshouses at the Boyce Thompson Institute, NY, USA, under standard conditions (27/19°C; 16-h light/8-h dark). Seed was obtained from the Tomato Genetics Resource Center at the University of California, Davis, CA, USA (http://tgrc.ucdavis.edu).

Carotenoid profiling by HPLC

Approximately 200 mg of frozen pericarp was used for carotenoid extraction.

Pooled samples of pericarp from the ILs and individual fruit (pericarp) from the transgenic plants were used for extraction. Carotenoids and chlorophyll were extracted, and their quantifications were performed as previously described (Alba *et al.*, 2005; Vrebalov *et al.*, 2009). Carotenoid composition was analyzed by HPLC (YMC Carotenoid S-5, 4.6 × 250 mm; Waters, http://www.waters.com) using a photodiode array detector (PDA-100; Dionex, http://www.dionex.com).

Ethylene measurements and treatments

Fruits were harvested at the stages indicated in the text and kept at room temperature for 2 h to reduce harvest stress. The ethylene production of the fruit was measured by sealing whole fruits in 250-ml airtight jars for 2 h at 22°C, taking 1 ml of headspace gas from the chamber and injecting it into a gas chromatograph (Hewlett-Packard 5890 series II; Hewlett-Packard, http://www.hp.com) fitted with a flame ionization detector and an activated alumina column. Ethylene concentrations were calculated using a 10-ppm ethylene standard (Airgas, Inc., http://www.airgas.com), and normalized by fruit mass.

For treatments with ethylene, tagged (35 days after anthesis) mature green fruits were exposed to 20 μL L^{-1} ethylene for 16 h in sealed 250-mL jars. The ethylene levels of the MG fruits were measured before ethylene treatment and while the internal color was fully green, but with the seeds fully developed, as per the definition of mature green fruit. A total of nine fruits were treated with ethylene and pooled into groups of three, yielding three biological replicates for RNA extraction and subsequent qRT-PCR analysis.

Transcriptome analysis

Total RNA for the microarray was obtained from pericarp tissues of ripening tomato fruit, and gene expression was compared between pairs of samples simultaneously. Tomato TOM2 long oligonucleotide arrays representing 12 000 unigene sequences were probed simultaneously with Cy5- and Cy3-labeled probes. Probe preparation, hybridization conditions and the washing protocol were performed as described previously (Alba *et al.*, 2004). Microarray images were analyzed to generate numerical data using ImaGene 5.5 (BioDiscovery, http://www.biodiscovery.com). Differentially expressed genes were identified using patterns from gene expression (Grant *et al.*, 2005). Functional annotations were assigned according to Alba *et al.* (2005).

Correlation analysis of genes and metabolites

Gene expression correlations between the 12 000 unigenes and nine carotenoids were calculated using the Pearson correlation coefficient using R. Any missing expression data was imputed by *k* nearest neighbor averaging ($k = 30$) (Troyanskaya *et al.*, 2001). The genes were selected by the criterion that they were correlated significantly with at least one carotenoid ($|r| > 0.5$; $P < 0.01$). The heat maps of carotenoid and carotenoid genes were generated by two-way hierarchical clustering using R. The map of functional categories was conduced using the *S. lycopersicum* mapping file of MapMan (http://gabi.rzpd.de/projects/MapMan). Genes selected by transcript–carotenoid correlation analysis were classified into functional BINs. The functional significance of BIN was estimated based on the correlation coefficients between the genes and each carotenoid. TF genes expressed in fruit were extracted from the digital expression analysis found in the TED database (Fei *et al.*, 2011). The correlation network for these TFs and carotenoids was generated using PAJEK 1.19 (Batagelj and Mrvar, 2002) (http://vlado.fmf.uni lj.si/pub/networks/pajek). The expression data from IL12-2

was excluded from correlation analysis with α-carotene, δ-carotene and lutein, as this IL harbors a known lycopene epsilon cyclase variant that could distort the results.

RNAi construct development and tomato transformation

A 316-bp *SlERF6*-specific DNA fragment located in the 3′ region of the cDNA (EST clone cLEG51D19) (i.e. bases 637–952 of GenBank accession JN616265) was amplified by PCR using *Pfu* Ultra DNA polymerase (Stratagene, now Agilent, http://www.genomics.agilent.com), the gene-specific primers SlERF6-attB1-F and SlERF6-attB2-R (see Table S6), and the EST clone as a template. The purified cDNA fragment was cloned into pHellsgate 2 (Wesley *et al.*, 2001) as an inverted repeat under the control of the *35S* promoter using the Gateway cloning system (Gateway BP Clonase Enzyme Mix; Invitrogen, http://www.invitrogen.com). Construct integrity was confirmed by sequencing and introduced into AC tomato by *Agrobacterium tumefaciens* 'LB4404' using previously described methods (Fillatti *et al.*, 1987). Plants that inherited the transgene were identified by PCR using CaMV *35S*-specific primers 35S-F and 35S-R (see Table S6), and by Southern blot analysis using a CaMV *35S*-specific probe.

Measuring mRNA by quantitative RT-PCR

Total RNA (2 μg) was reverse-transcribed with random hexamers and Superscript III (Invitrogen), according to the manufacturer's instructions. Purified cDNA (2 ng) was used as a template for qRT-PCR. qRT-PCR assays were carried out with gene-specific primers (Table S6), using an ABI PRISM 7900HT (Applied Biosystems, http://www.appliedbiosystems.com) real-time thermocycler and the SYBR Green PCR master mix (Applied Biosystems), according to the manufacturer's instructions. Relative template abundance was quantified using the relative standard curve method described in the ABI PRISM 7900HT manual, and the data were normalized for the quantity of *18S* RNA. A serial dilution of 0.4, 2, 10, 50 and 250 ng of each studied gene fragment was used to determine the amplification efficiency of each target and housekeeping gene. Duplicates from three biological replicates were used in two independent experiments.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Alignment and phylogenic analysis of *SlERF6* with other members of the AP2-ERF subgroup-VII transcription factors in tomato and Arabidopsis.

Figure S2. Fruit phenotype of SlERF6-RNAi lines and an azygous line.

Table S1. List of 953 genes correlated with carotenoid accumulation based on TOM2 transcriptome profiling.

Table S2. Previously described carotenoid and/or color-associated loci.

Table S3. Transcription factor candidates for carotenoid effects identified by transcriptome–metabolite network analysis.

Table S4. Days from anthesis to breaker stage in azygous and *SlERF6*-RNAi lines.

Table S5. Genes with expression changes ($n = 8$; $P < 0.05$) of at least twofold in the *SlERF6*-RNAi lines (BR fruit) from microarray (TOM2) analysis.

Table S6. Primer sequences.

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