The Plant Cell, Vol. 17, 2954–2965, November 2005, www.plantcell.org

Transcriptome and Selected Metabolite Analyses Reveal Multiple Points of Ethylene Control during Tomato Fruit Development [W]

Rob Alba,[a] Paxton Payton,[a,1] Zhanjun Fei,[a,2] Ryan McQuinn,[b] Paul Debbie,[a] Gregory B. Martin,[a,c] Steven D. Tanksley,[d] and James J. Giovannoni[a,b,3]

[a] Boyce Thompson Institute for Plant Research, Cornell University Campus, Ithaca, New York, 14853
[b] U.S. Department of Agriculture, Agricultural Research Service, Plant, Soil, and Nutrition Laboratory, Ithaca, New York, 14853
[c] Department of Plant Pathology, Cornell University, Ithaca, New York, 14853
[d] Department of Plant Breeding, Cornell University, Ithaca, New York, 14853

Transcriptome profiling via cDNA microarray analysis identified 869 genes that are differentially expressed in developing tomato (*Solanum lycopersicum*) pericarp. Parallel phenotypic and targeted metabolite comparisons were employed to inform the expression analysis. Transcript accumulation in tomato fruit was observed to be extensively coordinated and often completely dependent on ethylene. Mutation of an ethylene receptor (*Never-ripe* [*Nr*]), which reduces ethylene sensitivity and inhibits ripening, alters the expression of 37% of these 869 genes. *Nr* also influences fruit morphology, seed number, ascorbate accumulation, carotenoid biosynthesis, ethylene evolution, and the expression of many genes during fruit maturation, indicating that ethylene governs multiple aspects of development both prior to and during fruit ripening in tomato. Of the 869 genes identified, 628 share homology (E-value $\leq 1 \times 10^{-10}$) with known gene products or known protein domains. Of these 628 loci, 72 share homology with previously described signal transduction or transcription factors, suggesting complex regulatory control. These results demonstrate multiple points of ethylene regulatory control during tomato fruit development and provide new insights into the molecular basis of ethylene-mediated ripening.

INTRODUCTION

Fruit are unique to flowering plants and confer selective advantage to these species by aiding seed maturation and dispersal. The nutrients and colorful pigments contained in many fleshy fruits have been selected for this purpose through both evolution and domestication. Fleshy fruit are rich in flavor compounds, fiber, vitamins, and antioxidants and are integral components of animal diets (including humans). This biological and dietary significance has made the molecular dissection of fruit maturation and ripening an area of considerable research interest (reviewed in Alexander and Grierson, 2002; Bramley, 2002; Giovannoni, 2004).

Fruit development occurs in five stages, including organogenesis, expansion, maturation, ripening, and senescence. In climacteric fruit, ripening is preceded by a dramatic increase in C_2H_4 evolution (Burg and Burg, 1962), which remains at basal levels prior to the onset of ripening (Herner and Sink, 1973; Lincoln and Fischer, 1988; Klee, 1993; Lashbrook et al., 1998). In fruit from tomato (*Solanum lycopersicum*) cv Ailsa Craig, maximum levels of C_2H_4 production correlate with rapid accumulation of β-carotene and *trans*-lycopene in this tissue, which occurs 1 to 5 d after the breaker stage of ripening (Grierson and Tucker, 1983; Giovannoni et al., 1989; Balbi and Lomax, 2003). Our analyses of Ailsa Craig fruit (R. Alba and J.J. Giovannoni, unpublished data) consistently indicate patterns of ethylene evolution that corroborate published data for this cultivar, indicating a dramatic increase in ethylene production concurrent with the breaker stage, increasing for the first 4 to 7 d of ripening and then dropping to ~25 to 50% of maximal levels over the next week (Grierson and Tucker, 1983; Giovannoni et al., 1989; Balbi and Lomax, 2003). The discovery that mutation of the Nr^+ C_2H_4 receptor prevents ripening in tomato via ethylene insensitivity even when ethylene is applied exogenously (as the mutation results in greatly reduced endogenous ethylene synthesis) demonstrates that climacteric C_2H_4 signaling plays a central role in coordinating the molecular processes required for ripening (Lanahan et al., 1994; Wilkinson et al., 1995; Yen et al., 1995). Furthermore, numerous studies indicate that C_2H_4, via Nr^+, exerts part of its influence on fruit ripening via transcriptional control of genes associated with carotenoid metabolism, ascorbate accumulation, fruit softening, aroma, and C_2H_4 synthesis (Biggs and Handa, 1988; Lincoln and Fischer, 1988; DellaPenna et al., 1989; Fray and Grierson, 1993; Fraser et al., 1994; Yen et al., 1995).

[1] Current address: USDA, Agricultural Research Service, Cropping Systems Research Laboratory, Lubbock, TX 79415.
[2] Current address: Virginia Bioinformatics Institute, Blacksburg, VA 24060.
[3] To whom correspondence should be addressed. E-mail jjg33@cornell.edu; fax 607-254-2958.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: James J. Giovannoni (jjg33@cornell.edu).
[W] Online version contains Web-only data.
Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.105.036053.

Six different C_2H_4 receptors have been identified in the tomato genome, all of which are differentially expressed in various tissues (Lashbrook et al., 1998). Transcripts encoding two C_2H_4 receptors (*Never-ripe* [*Nr*] and *ethylene-response 4*) accumulate to high levels in ripening tomato fruit, suggesting these two receptors may function in C_2H_4-induced ripening (Wilkinson et al., 1995; Yen et al., 1995). It is also known that tomato ripening is inhibited by a fruit-localized mitogen-activated protein kinase cascade and that C_2H_4, probably via the putative kinase encoded by *CONSTITUTIVE TRIPLE RESPONSE1*, deactivates this signaling cascade allowing ripening to proceed (Tieman et al., 2000). Thus, as in *Arabidopsis thaliana* seedlings, C_2H_4 receptors in tomato fruit act as negative regulators of C_2H_4 signaling (Klee, 2002).

Work in this area has now shifted to regulation of climacteric C_2H_4 (Thompson et al., 1999; Vrebalov et al., 2002), C_2H_4 receptors other than Nr^+ (Lashbrook et al., 1998), C_2H_4 signaling factors (Tieman et al., 2001; Leclercq et al., 2002; Adams-Phillips et al., 2004), and other coordinators of ripening (Bauchot et al., 1998; Alba et al., 2000). Together, these data outline a minimal regulatory network for tomato ripening, including a significant component influenced by C_2H_4 (Giovannoni, 2004).

Despite such advances, little is known about transcriptome dynamics during expansion, maturation, or ripening of climacteric fruit. Here, we identify 869 genes that are differentially expressed in tomato pericarp, demonstrate that 37% of these genes are regulated by C_2H_4, and show that transcript accumulation is extensively coordinated in tomato pericarp. We show that C_2H_4 regulates biochemistry, morphology, and transcriptome activity throughout fruit development. Finally, comparison of expression patterns for genes encoding enzymes in fruit development pathways to the measured products of said pathways (e.g., ethylene, carotenoids, and ascorbate) suggests novel fruit development regulatory points influenced by ethylene.

RESULTS

Differential Expression in Developing Tomato Pericarp

To identify differentially expressed genes in tomato pericarp, we used the TOM1 cDNA microarray and a time-series loop design. The time-series loop included 10 stages of tomato fruit development that were compared using sequential direct comparisons and triple dye-flip replication (Figure 1). Differential transcript accumulation was documented using two-color hybridizations to the TOM1 array, which contains 12,899 different EST clones representing ~8500 tomato genes. Array fabrication, and the EST libraries from which the TOM1 array is derived, have been described previously (van der Hoeven et al., 2002; Alba et al., 2004).

Analysis of variance (ANOVA; $P < 0.05$, false discovery rate [FDR] = 0.05) indicated that 869 (10.1%) genes represented in the TOM1 array are differentially expressed at one or more assayed stages during tomato pericarp development (see Supplemental Table 1 online). Assuming the TOM1 array represents 25% of the functional tomato genes (van der Hoeven et al., 2002), this implies that 3476 genes are differentially expressed during tomato fruit development.

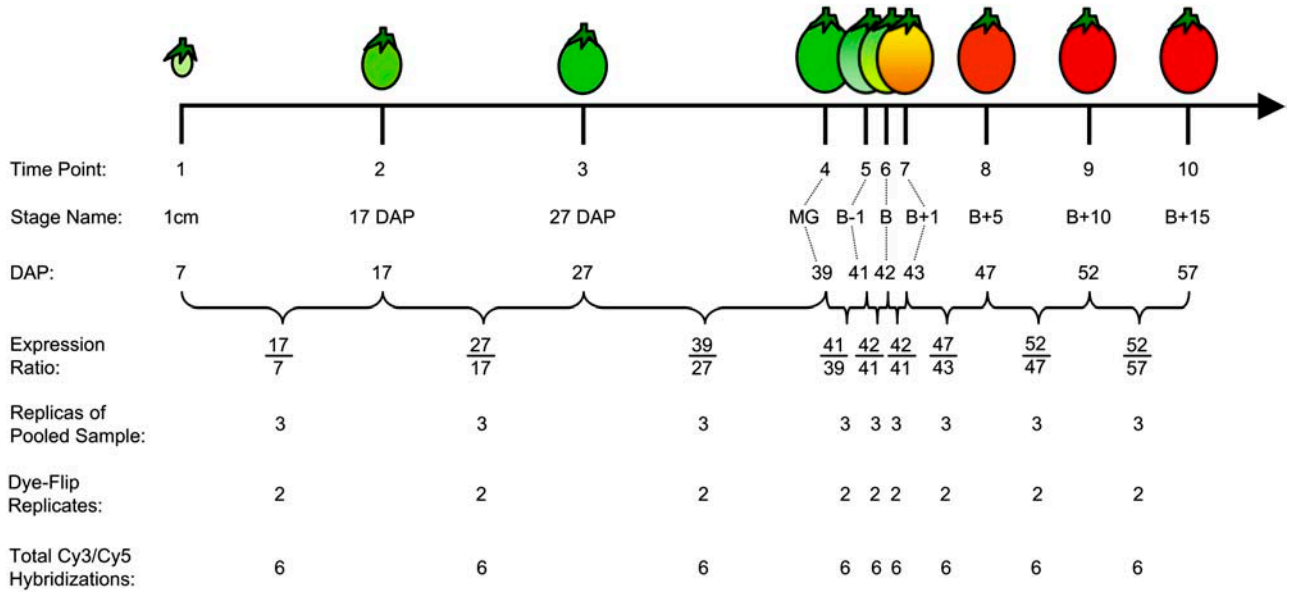

Figure 1. Experimental Design.

For each of the nine sequential time-point comparisons, Cy-labeled cDNAs were hybridized to six independent microarrays using a triple dye-flip design. Equivalent age fruit tissue was collected and pooled from a normal line (cv Ailsa Craig) and a line nearly isogenic and homozygous for the *Nr* mutation. Cy3, cyanine3 fluor; Cy5, cyanine5 fluor; B, breaker stage; B+5, 5 d after breaker; B+10, 10 d after breaker; B+15, 15 d after breaker; DAP, days after pollination; MG, mature green.

To verify their identity, the EST clones for 607 of these 869 genes have been resequenced in both the 5′ and 3′ directions; the remaining 262 EST clones have been resequenced in only the 5′ or 3′ direction or remain to be resequenced successfully. Only those genes for which both ends had been resequenced were utilized for the following analysis. Functional categorization of the 607 5′/3′ sequence-verified ESTs is shown in Table 1 and indicates that fruit development involves many loci encoding translational machinery, transcription factors, and signal transduction components, in addition to genes associated with primary metabolism, photosynthesis, cell wall metabolism, and hormone responses. The fact that 73 of these 869 loci share homology with signal transduction or transcription factors indicates that tomato fruit development requires complex regulatory control.

Comparative Analysis of Wild-Type and *Nr* Fruit

The 6254 EST features in the TOM1 array yielded complete expression profiles for both wild-type and *Nr* fruit and allow one to compare fruit transcriptome dynamics in the context of normal versus inhibited ethylene responses (Figures 2 to 4). The *Nr* lesion has a significant effect on the fruit transcriptome prior to ripening. Figure 2 shows ~2000 TOM1 features (i.e., array elements 2750 to 4750), corresponding to at least 1250 unigenes, with maturation-related expression patterns that are dependent on C_2H_4 perception via Nr^+. Figure 2 also indicates that Nr^+ is a negative regulator of expression for many genes in the pericarp and confirms that ripening-related expression is not always dependent on C_2H_4 (Vrebalov et al., 2002). Nonetheless, we cannot exclude the possibility that some ripening-related expression in *Nr* fruit is regulated by C_2H_4 because severity of the *Nr* phenotype is genotype dependent and residual C_2H_4 responsiveness has been documented in cv Ailsa Craig (Lanahan et al., 1994; Yen et al., 1995).

Mutation of *Nr* also influences developmental and biochemical processes that occur prior to ripening (see Supplemental Table 2 and Supplemental Figure 1 online). Mature green *Nr* fruit are longer (with respect to fruit width; $P < 0.0001$), have thicker outer pericarp ($P < 0.0001$), fewer locules ($P < 0.0001$), fewer mature seeds ($P < 0.0001$), and accumulate more reduced ascorbate (ASC).

Figure 3 shows nine expression patterns observed in tomato pericarp and demonstrates that gene expression in wild-type fruit is highly coordinated during development. Coordinated expression among functionally related genes is also evident (e.g., cytosolic ribosome genes; Figure 3, Ribo). As expected, most of the prominent expression patterns observed in this study correlate well with processes such as fruit expansion, maturation, and ripening (Figure 3, left). Furthermore, transcriptome activity in tomato pericarp is highly dependent on C_2H_4 perception via Nr^+ (Figure 3, right), and in some cases this hormone regulates the coordinated expression of functionally related loci (e.g., cytosolic ribosome genes; Figure 3, Ribo).

To better understand C_2H_4-regulated transcriptome activity in this tissue, expression patterns for the 869 genes that are differentially expressed in wild-type fruit (cv Ailsa Craig) were compared with their expression patterns in *Nr* fruit. This comparison indicates that >37% of these genes were influenced by the *Nr* lesion (Pearson correlation, $r < 0.25$). Because the *Nr* allele investigated causes a more severe phenotype in cv Pearson than it does in cv Ailsa Craig, a limited number of stages (B, B+3, and B+10) of *Nr* fruit from cv Pearson were profiled for verification purposes. This experiment shows that a minimum of 392 genes, and perhaps as many as 739 genes, have Nr^+-dependent transcript accumulation during the three stages investigated; putative identities of these 739 genes are listed in Supplemental Table 3 online. These data, together with those shown in Figures 2 and 3 and Supplemental Figure 1 and Supplemental Table 2 online, demonstrate that C_2H_4 is a key determinant of transcriptome activity in tomato pericarp throughout fruit development and including stages prior to ripening.

Of the 607 genes described above, 72 (12%) were assigned to the categories transcription factor (36 loci, E-value $<1 \times 10^{-10}$) and signal transduction (36 loci, E-value $<1 \times 10^{-10}$). Figure 4 depicts expression for 10 candidate regulatory genes, including six putative transcription factors: ASC oxidase promoter binding protein (AOBP), basic leucine zipper1 (bZIP1), bZIP61, carbon catabolite repressor-associated factor (CAF), multiprotein bridging factor 1 (MBF1), and transcription factor IIE (TFIIE). All of the genes shown in Figure 4 (and many of the candidate regulatory genes in Supplemental Table 1 online) are under C_2H_4 control in developing tomato pericarp. Candidate regulatory genes likely to encode signal transduction factors have also been identified. Four examples of genes belonging to this functional category are shown in Figure 4, including a putative mitogen-activated protein kinase kinase (MAPKK) that is negatively regulated by C_2H_4 during ripening. Thirty-two other genes assigned to this functional category are shown in Supplemental Table 1 online.

Table 1. Functional Classification of 607 Differentially Expressed Genes[a]

Assignment	No. of Genes	Percentage of Total
Cell transport	7	1.15
Secondary metabolism	8	1.32
Membrane transport	11	1.81
Energy pathways[b]	12	1.98
Cell structure	16	2.64
Stress responses	20	3.29
Defense responses	21	3.46
Hormone responses	24	3.95
Protein degradation	26	4.28
Cell wall	29	4.78
Photosynthesis	36	5.93
Signal transduction	36	5.93
Transcription factor	37	6.10
Primary metabolism	38	6.26
Protein biosynthesis	39	6.43
Other	110	18.12
Unknown protein	137	22.57
Total	607	100.00

[a] One-way ANOVA ($P < 0.05$, FDR = 0.05).
[b] Does not include photosynthesis genes.

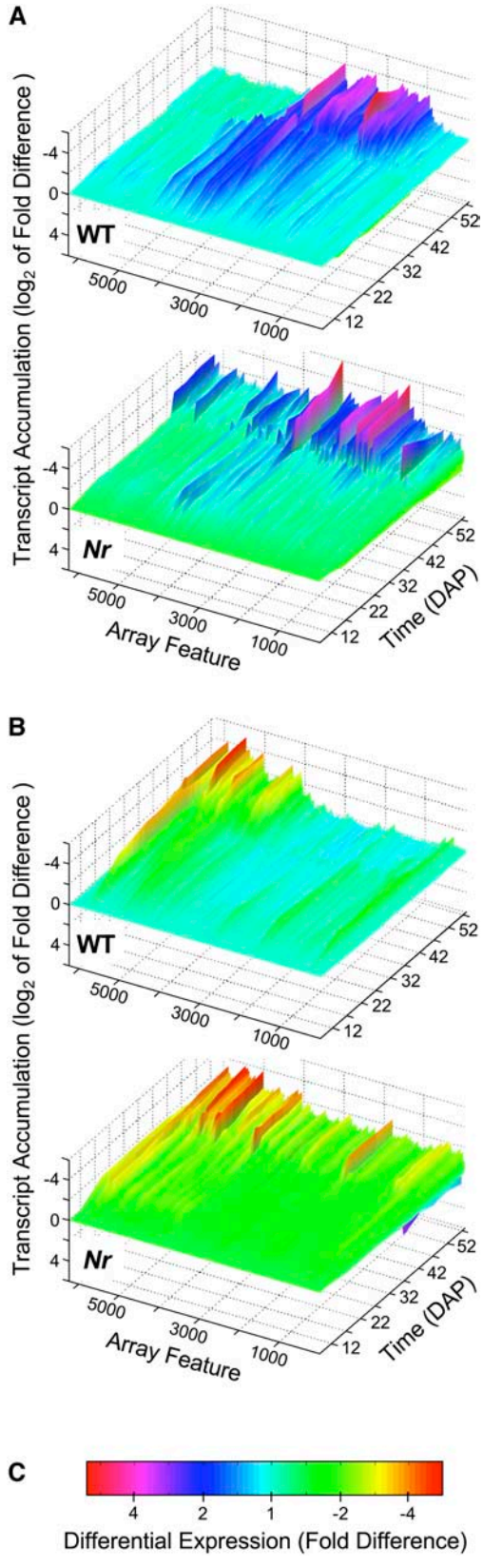

Figure 2. Effect of the *Nr* Lesion on Transcriptome Dynamics.

Transcriptional Regulation of C_2H_4 Biosynthesis

The TOM1 array allowed profiling of six enzymes thought to influence C_2H_4 biosynthesis in tomato pericarp (Figure 5A), including threonine synthase (TS), cystathionine γ-synthase (CGS), methionine sulfoxide reductase (E4 and MSR), *S*-adenosyl-L-methionine synthase1 (SAM1), 1-aminocyclpropane-1-caroxylic acid synthase (ACS2 and ACS4), and 1-aminocyclopropane-1-carboxylic acid oxidase1 (ACO1). In wild-type fruit (Figure 5B, left), expression of *TS* is not ripening related and shows a steady decline throughout fruit development. *CGS* transcripts accumulate gradually during fruit maturation to their maximum level immediately prior to ripening. Maximum transcript accumulation for *CGS* at 41 d after pollination (DAP) in this tissue is confirmed by RNA gel blot analysis (see Supplemental Figure 2 online). *E4* transcripts, thought to encode an MSR required for climacteric C_2H_4 synthesis (Alexander and Grierson, 2002), accumulate during fruit maturation and the early stages of ripening, while a second *MSR* is expressed preferentially during the final stages of ripening (Figure 5B, left). *SAM1*, *ACS2*, *ACS4*, and *ACO1* are regulated by different mechanisms in wild-type tomato pericarp. *SAM1* transcripts accumulate gradually during fruit maturation and are rapidly downregulated prior to the breaker stage of pericarp development. Unlike *SAM1*, *ACS2* and *ACS4* are upregulated at the breaker stage and reach maximum expression levels 5 d after ripening begins. In wild-type fruit, *ACO1* transcripts accumulate gradually during fruit maturation, reach maximum levels shortly after the breaker stage, and show a steady decline during ripening (Figure 5B, left).

Nr does not influence the pattern of *TS* expression in this tissue but may influence the extent to which *TS* is downregulated (Figure 5B, right). In addition, these data show that two *MSR* genes are under C_2H_4 control. As shown by Barry et al. (2000), *ACS4* is expressed in a ripening-related manner irrespective of the *Nr* lesion (Figure 5B, right). Contradictory to Barry et al. (2000), these data also imply that ripening-related expression of *ACS2* in this tissue is not eliminated by the *Nr* lesion. One explanation for this discrepancy is that the *Nr* allele is slightly leaky in the Ailsa Craig cultivar (Lanahan et al., 1994), whereas the data reported by Barry et al. (2000) was acquired using cv Pearson. The *Nr* lesion has two effects on *ACO1* expression in this tissue: immature green fruit (i.e., 17 DAP) show decreased levels of this transcript as compared with the wild type, while maximum levels are delayed 4 d in ripening *Nr* fruit (Figure 5B, right).

Transcriptional Regulation of Carotenoid Accumulation

The TOM1 array allows profiling of six genes known to influence carotenoid biosynthesis in tomato pericarp (Figure 6A), including

Nr^+ has a global effect on expression in tomato pericarp and regulates thousands of genes prior to ripening. These data also indicate that many ripening-related genes are not influenced by the *Nr* allele in this cultivar. Two different perspectives of each transcriptome are shown: **(A)** and **(B)** show relative gene activation and gene repression, respectively. **(C)** is a color map depicting relative transcript accumulation in **(A)** and **(B)**. DAP, days after pollination.

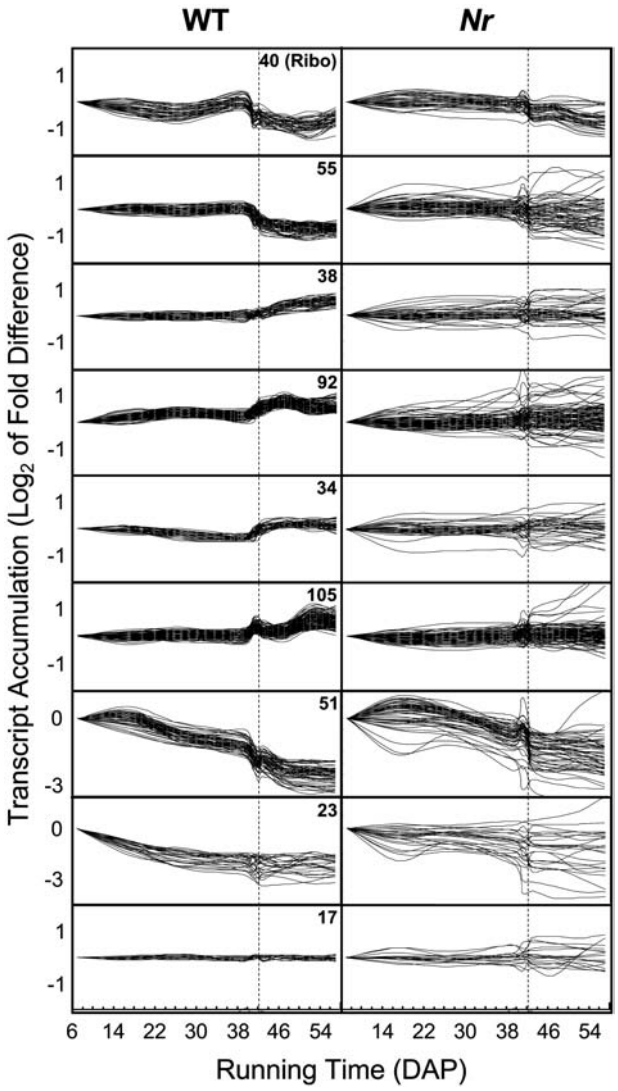

Figure 3. Coordinated Expression during Fruit Development.

Transcriptome profiling in wild-type fruit (left) reveals highly coordinated expression in developing pericarp. In some cases, this coordination correlates with gene function (e.g., 40 cytosolic ribosome genes, top panel). Comparison of wild-type and *Nr* fruit (right) shows that C_2H_4 dictates the expression of many ripening-related genes. Each trace represents a different gene, and the number of genes shown in each panel is noted in the wild-type plot; genes shown for the *Nr* tissue are the same genes shown for the wild-type tissue. The vertical dashed line denotes the breaker stage of fruit development. DAP, days after pollination; Ribo, cytosolic ribosome genes.

1-deoxy-D-xylulose 5-phosphate synthase (*DXS*), phytoene synthase 1 (r^+), phytoene desaturase (pds^+), plastid terminal oxidase (gh^+), lycopene β-cyclase (B^+), and β-carotene hydroxylase (*CrtR-b*$^+$). Results acquired in this study (Figure 6B, left) confirm previous reports about the expression of *DXS*, r^+, pds^+, gh^+, and B^+ in wild-type tomato fruit (Giuliano et al., 1993; Fraser et al., 1994; Pecker et al., 1996; Lois et al., 2000; Isaacson et al., 2002; Barr et al., 2004). Figure 6B (left) also shows the expression of *CrtR-b*$^+$ in this tissue, which has not been reported previously. Similar to *DXS*, r^+, pds^+, and gh^+, *CrtR-b*$^+$ transcripts accumulate immediately prior to and during pericarp ripening. Ripening-related expression of *DXS*, pds^+, gh^+, and B^+ is not observed in fruit harboring the *Nr* mutation (Figure 6B, right), demonstrating that C_2H_4 perception by Nr^+ governs expression of numerous carotenoid biosynthetic genes in tomato pericarp. Nr^+ does not regulate expression of r^+ and *CrtR-b*$^+$, indicating that some carotenoid genes are expressed in a ripening-related C_2H_4-independent manner (Figure 6B, right).

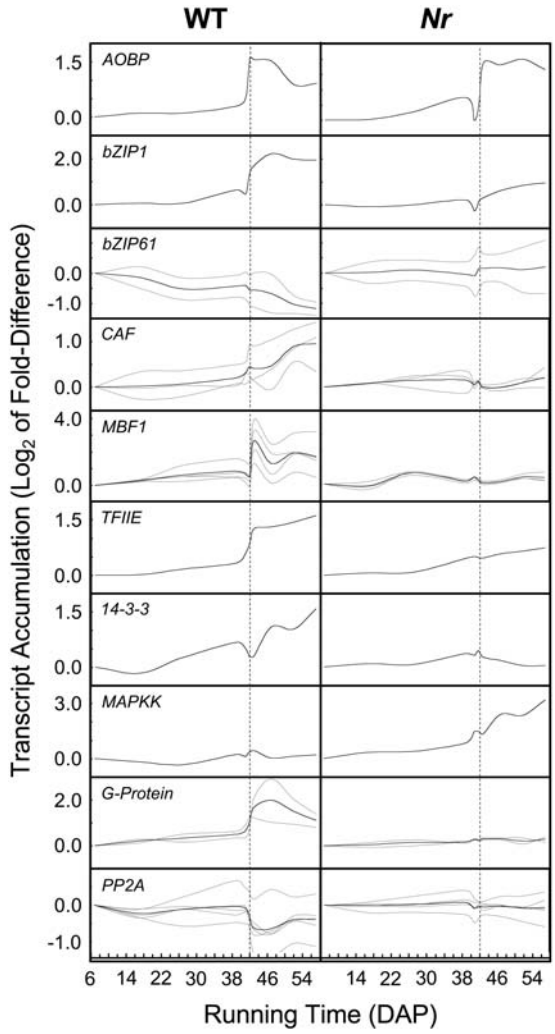

Figure 4. Candidate Regulatory Genes for Tomato Ripening.

Ten of the seventy-two candidate regulatory genes identified in this study are shown. Gray lines show pseudoreplicates in the TOM1 array for the gene shown; black lines show a running mean when pseudoreplicates exist or expression of the gene shown if no pseudoreplicates exist. Only ESTs with substantial sequence homology were considered (E-value $<1 \times 10^{-10}$), and all EST clones shown have been sequence verified. The vertical dashed line denotes the breaker stage of fruit development. 14-3-3, 14-3-3 domain; DAP, days after pollination; G-protein, transducin-like heterotrimeric G-protein; PP2A, Ser/Thr protein phosphatase 2A regulatory chain.

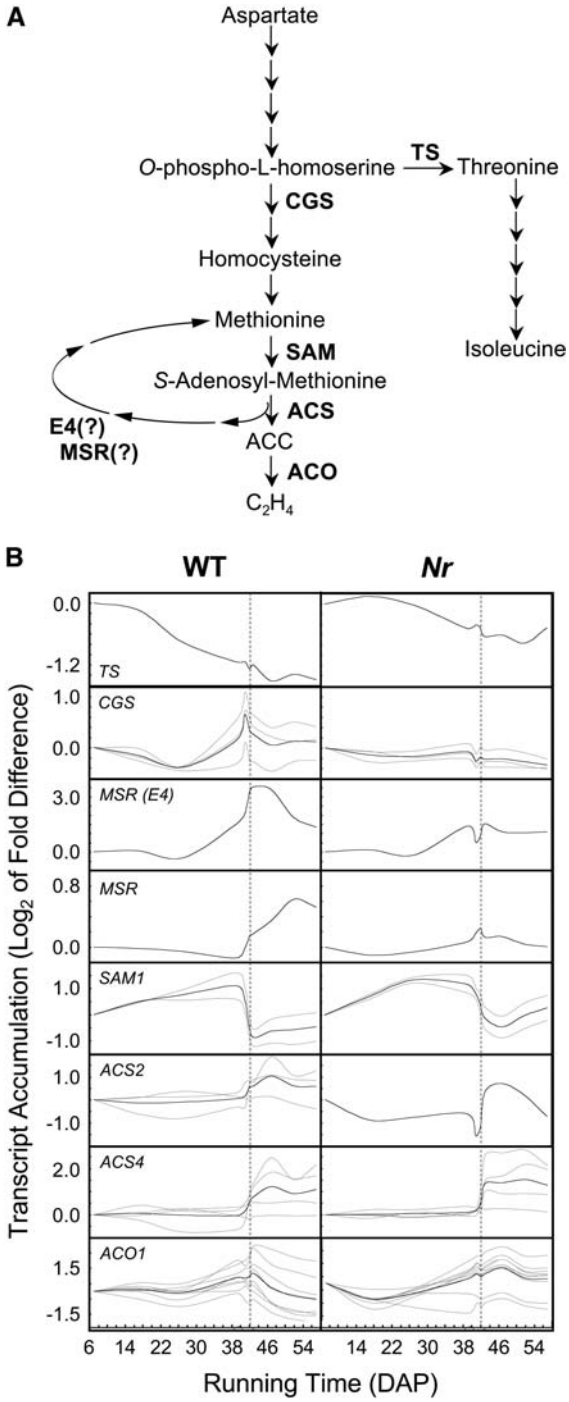

Figure 5. Transcriptional Regulation of C_2H_4 Biosynthesis.

Seven genes thought to encode C_2H_4 biosynthetic enzymes are expressed in a ripening-related manner, five of which are under C_2H_4 control.

(A) Pathway of C_2H_4 biosynthesis in tomato.

(B) Expression profiles for *TS*, *CGS*, two likely *MSR* homologs, *SAM1*, *ACS2*, *ACS4*, and *ACO1*. Gray lines show pseudoreplicates in the TOM1 array for the gene shown; black lines show a running mean when pseudoreplicates exist or expression of the gene shown if no pseudoreplicates exist. Only ESTs with substantial sequence homology were considered (E-value $<1 \times 10^{-10}$), and all EST clones have been sequence verified. The vertical dashed line denotes the breaker stage of fruit development. E4, ripening-related protein E4.

Figure 7A depicts the accumulation of phytoene (PE), phytofluene (PF), lycopene (Lyc), γ-carotene (γ-Car), and β-carotene (β-Car) in wild-type tomato pericarp. Lyc content in wild-type pericarp at 57 DAP was 91.6 (±1.08) μg/g fresh weight (FW), and β-Car content in this tissue was 9.3 (±0.35) μg/g FW. In wild-type fruit, β-Car and γ-Car begin accumulation 2 d prior to PE, PF, or Lyc, and β-Car reaches its maximum 10 d earlier than PE, PF, Lyc, and γ-Car in this tissue. Lyc content in *Nr* pericarp at 57 DAP was 9.6 (±0.70) μg/g FW, which is ~10% of that detected in wild-type pericarp at 57 DAP (Figure 7B). These data also indicate that the *Nr* lesion causes a similar reduction in the accumulation of PE, PF, and γ-Car, which only reach 10 to 20% of their wild-type levels in *Nr* fruit. The *Nr* lesion appears to have little effect on the net accumulation of β-Car in this tissue. Although delayed in *Nr* pericarp, this tissue accumulates 89% (8.3 μg/g FW) of the β-Car produced in equivalent tissue from wild-type fruit (Figure 7B), suggesting that conversion of carotenoid precursors to β-Car is C_2H_4 independent in this tissue. Together, these results confirm that carotenogenesis in tomato pericarp is, at least in part, governed by C_2H_4-dependent transcriptional regulation. These results also demonstrate that C_2H_4 dictates the accumulation of carotenoids in this tissue, including the metabolic shift from β-Car to Lyc.

DISCUSSION

The *Nr* Lesion and Transcriptome Dynamics in Tomato Pericarp

It is estimated that the tomato genome contains ~35,000 genes (van der Hoeven et al., 2002). Extrapolating from our array data, Supplemental Table 1 online suggests that ~3476 genes in the tomato genome (i.e., 10.1% of the gene space) are differentially expressed at some point in developing pericarp tissue (ANOVA, $P < 0.05$, FDR = 0.05). Two arguments suggest that this is an underestimate of the total differential gene expression in developing tomato fruit. First, the experimental design employed here cannot detect differential expression prior to 7 DAP (i.e., during organogenesis), after the B+15 stage (i.e., fruit senescence), or uniquely between the stages assayed. Second, gene expression was monitored in outer pericarp tissue and does not account for transcriptome activity in other fruit tissues (e.g., columella, locular jelly, and seeds).

Essentially all of what is known about the role of Nr^+ in fruit development pertains to the molecular basis of tomato ripening, and little is known about the function of this receptor during preripening fruit development. The data reported here show that C_2H_4, via Nr^+, influences the expression of thousands of genes in green fruit prior to the onset of ripening. In addition, we show that this hormone and receptor govern morphological and biochemical processes, such as fruit shape (i.e., length-to-width ratio),

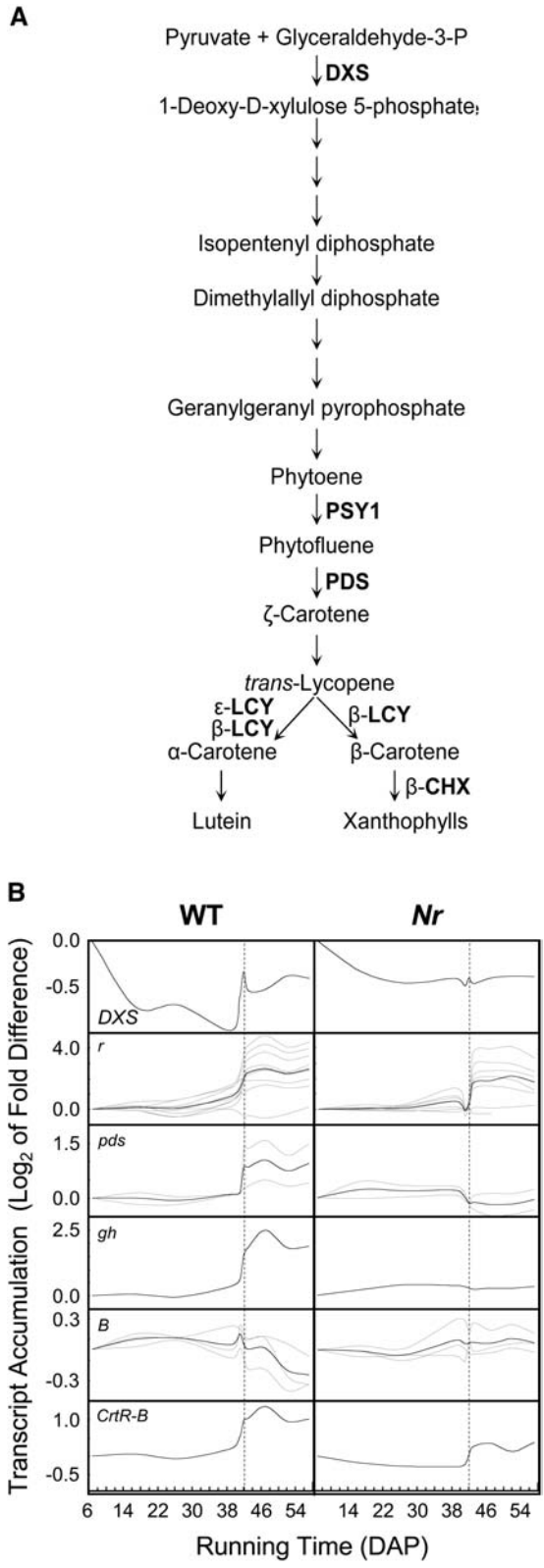

Figure 6. Transcriptional Regulation of Carotenoid Biosynthesis.

Six genes thought to be required for carotenoid biosynthesis are expressed in a ripening-related manner, four of which are under C_2H_4 control.

(A) Pathway of carotenoid biosynthesis in tomato.

(B) Expression profiles for *DXS*, r^+, pds^+, gh^+, B^+, and *CrtR-b*$^+$. Gray lines show pseudoreplicates in the TOM1 array for the gene shown; black lines show a running mean when pseudoreplicates exist or expression of the gene shown if no pseudoreplicates exist. Only ESTs with substantial sequence homology were considered (E-value $<1 \times 10^{-10}$), and all EST clones have been sequence verified. The vertical dashed line denotes the breaker stage of fruit development.

pericarp thickness, locular development, and ASC accumulation. The *Nr* lesion also has adverse effects on seed production and/or seed development in mature green fruit, indicating that this locus may be a determinant of fecundity in this species. These results clearly indicate that C_2H_4 action in tomato pericarp is not limited to processes strictly associated with climacteric ripening. Instead, this hormone and receptor govern biochemical, physiological, and developmental processes throughout tomato fruit development, including those that occur prior to ripening (Figure 8).

Candidate Regulatory Genes for Fruit Development

This study identifies 72 candidate regulatory factors, many of which are likely to be regulators of specific genes or gene sets. The (putative) transcription factors identified belong to two distinct categories of transcriptional regulatory proteins. Genes such as *AOBP*, *bZIP1*, and *bZIP61* are homologous to sequence-specific DNA binding proteins thought to function as *trans*-acting factors in regulating gene expression. Genes such as *CAF*, *MBF1*, and *TFIIE* are transcriptional coactivators that comprise part of the RNA polymerase II complex (i.e., *TFIIE*) or exhibit protein–protein interactions with this complex (i.e., *CAF* and *MBF1*).

AOBP encodes a Dof/Zn-finger transcription factor that is proposed to repress *AOX* transcription and cause the accumulation of reduced ASC (Kisu et al., 1998; Shimofurutani et al., 1998). In contrast with these reports, our transcriptome and selective metabolite analyses show that *AOBP* expression is not positively correlated with ASC accumulation in tomato pericarp (Figure 4; see Supplemental Figure 1 online) and therefore imply that this metabolite is governed by factors in addition to or other than AOBP in this tissue. Two candidate bZIP proteins (bZIP1 and bZIP61) were also identified. bZIP1 has been cloned previously from tomato leaf tissue and is rapidly induced by wounding (Stanković et al., 2000). Nothing is known about the function of bZIP1 or bZIP61 in tomato fruit, but it is interesting to note that a bZIP1 homolog in tobacco (basic leucine zipper F) is induced by C_2H_4 in leaf tissue (Yang et al., 2001), and a bZIP61 homolog in tobacco and rice (*Oryza sativa*) encodes a phloem-specific transcription factor (Yin et al., 1997; Jakoby et al., 2002).

Data pertaining to CAF, MBF1, and TFIIE raise new possibilities about the role of transcriptional coactivators in hormonal coordination of plant transcriptomes. These proteins are particularly interesting because (1) transcript accumulation for these proteins is associated with fruit ripening, (2) their expression in

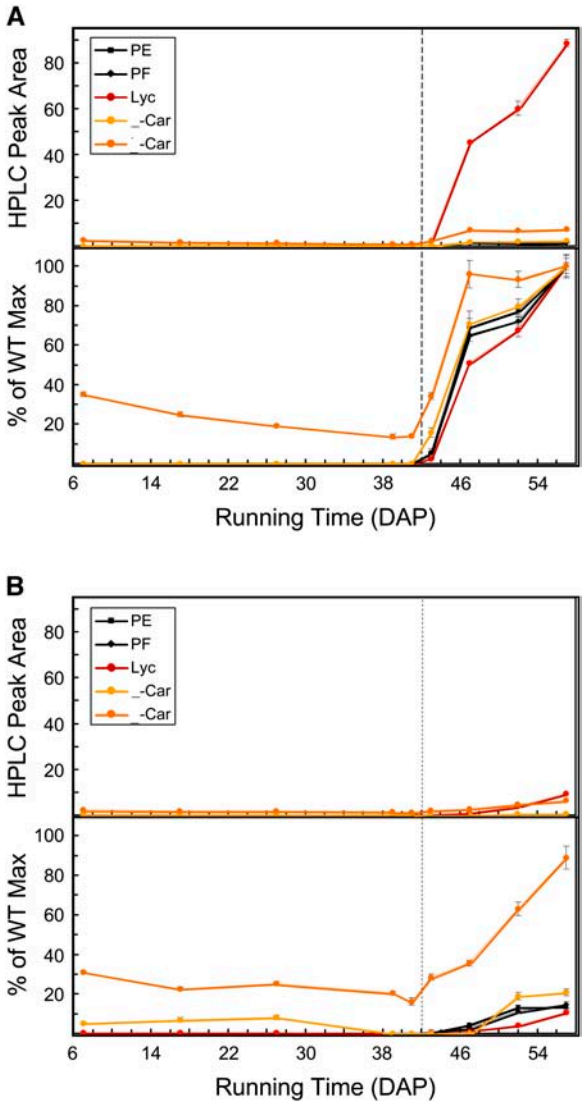

Figure 7. Carotenoid Metabolite Profiling in Tomato Fruit.

Ethylene regulates carotenoid metabolism in developing tomatoes. Carotenoid metabolite profiles for wild-type **(A)** and *Nr* **(B)** pericarp are shown. Carotenoid content (top panels) was determined from mean HPLC peak areas ($n = 8$, ±SE). Mean *trans*-lycopene and β-Car content at 57 DAP in wild-type fruit was 91.6 ± 1.076 μg/g FW and 9.3 ± 0.350 μg/g FW, respectively. Mean *trans*-lycopene and β-Car content at 57 DAP in *Nr* fruit was 9.6 ± 0.702 μg/g FW and 8.3 ± 0.526 μg/g FW, respectively. The vertical dashed line denotes the breaker stage of fruit development.

tomato pericarp is C_2H_4 regulated, and (3) these proteins govern genome-scale transcriptome dynamics in other species. For example, in yeast (*Saccharomyces cerevisiae*), CAF1 acts as a global repressor of transcript accumulation via interactions with TFIID, and this protein–protein interaction may govern the expression of large numbers of genes (Denis and Malvar, 1990; Badarinarayana et al., 2000). In *Drosophila* and silk worm (*Bombyx mori*), MBF bridges protein–rotein interactions between hormone receptors and the TATA-box binding protein (Li et al., 1994; Kabe et al., 1999), thus providing a direct molecular link between specific hormone signals and transcriptome activity in these species. In plants, MBF1 is under C_2H_4 control in species such as tomato and potato (*Solanum tuberosum*; Zegzouti et al., 1999; Godoy et al., 2001), but this is not the case in *Arabidopsis* seedlings (Tsuda et al., 2004). Furthermore, the fact that MBF1 is

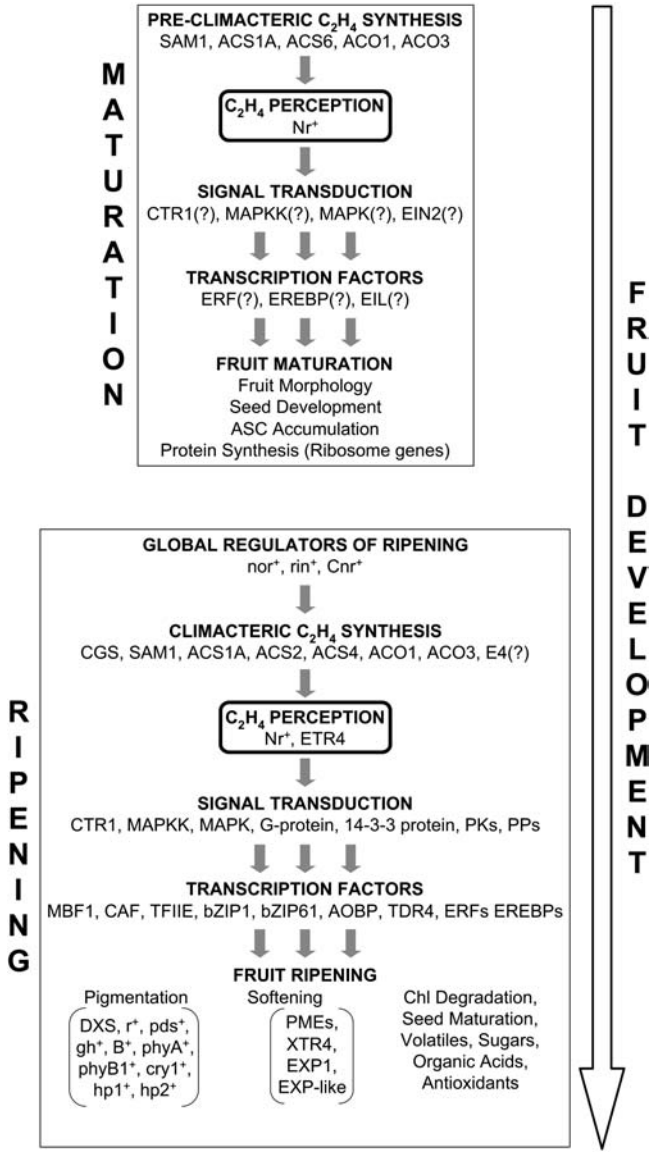

Figure 8. Model for C_2H_4 Regulatory Control in Tomato Fruit.

Time-series transcriptome analysis, morphometric analysis, and selective metabolite profiling reveal novel regulatory points in pathways associated with ripening and identify numerous candidate regulatory loci. Only a fraction of the candidate regulatory genes identified in this study are shown here. A complete list of C_2H_4-regulated genes, candidate regulatory factors, Sol Genomics Network (SGN) unigene identifiers, and corresponding expression profiles are included in Supplemental Tables 1 and 3 online.

associated with plant defense responses in potato, tobacco, and *Arabidopsis* (Godoy et al., 2001; Matsushita et al., 2002) suggests that this factor may govern transcriptome dynamics in unrelated plant processes (e.g., ripening and pathogen defense).

In addition to transcription factors, this project identified numerous (putative) signaling factors that are transcriptionally associated with fruit maturation and ripening. Four of these candidate regulatory loci are likely to encode a 14-3-3 protein, a MAPKK, a G-protein with homology to transducin, and a protein phosphatase regulatory subunit. The fact that these genes show C_2H_4-dependent expression suggests that they act downstream of C_2H_4 perception via Nr^+. The experimental system employed here identifies genes in other functional categories as well. For example, eight genes likely to encode proteins involved in cell wall metabolism have ripening-related expression profiles; four of these genes are under C_2H_4 control and have not been identified previously as differentially expressed in developing fruit (see Supplemental Table 1 online).

New Insights about C_2H_4 and Carotenoid Biosynthesis in Tomato Fruit

Climacteric C_2H_4 biosynthesis includes (1) conversion of Asp to Met, (2) conversion of Met to C_2H_4, and (3) the Met recycling pathway (Yang and Hoffman, 1984). Conversion of Met to C_2H_4 is, at least in part, governed via transcriptional regulation of *SAM*, *ACS*, and *ACO* gene family members; however, these genes are regulated by different mechanisms. *SAM1* transcripts accumulate preferentially in tomato pericarp during fruit maturation, while *ACS2*, *ACS4*, and *ACO1* transcripts accumulate concomitantly with C_2H_4 biosynthesis and pericarp ripening. Based on the expression data presented here, it is apparent that transcriptional regulation may govern other stages of the C_2H_4 biosynthetic pathway as well. For example, *TS* expression decreases throughout pericarp development, while *CGS* expression increases during the transition from a mature green fruit to a ripening fruit in an *Nr*-dependent manner. These transcriptional changes could influence TS and CGS and shift carbon flux from the Thr pathway to the C_2H_4 pathway during fruit ripening. Thus, the metabolic branch point comprised of TS and CGS, both of which compete for the substrate *O*-phospho-L-homoserine, appears to be a site of regulation for C_2H_4 biosynthesis in tomato pericarp and provides further explanation, in concert with the Yang cycle, for diversion of carbon flux to ethylene synthesis during climacteric ripening (see Figure 5A).

Regarding carotenoids, these results confirm previous reports showing that r^+, pds^+, and gh^+ are expressed in a ripening-related manner in tomato (Giuliano et al., 1993; Fraser et al., 1994; Pecker et al., 1996; Lois et al., 2000; Isaacson et al., 2002; Barr et al., 2004). Furthermore, the facts that r^+ expression is not influenced by the *Nr* lesion, while carotenoid accumulation is clearly dependent on Nr^+, indicate that r^+ expression is not sufficient to activate and maintain carotenoid metabolism in this tissue. The fact that r^+ is not influenced by the *Nr* lesion is also interesting because PE accumulation is delayed by approximately 5 d and reduced by ~87% in ripe pericarp from the *Nr* mutant. Thus, PE accumulation in tomato pericarp is not determined solely by r^+ expression. More likely, PE accumulation is determined by the expression of multiple genes in the carotenoid pathway, precursor availability, and/or posttranscriptional regulation of r^+ (Al-Babili et al., 1996). Although not the case with r^+, the *Nr* lesion abolishes ripening-related expression of pds^+ and gh^+, suggesting that desaturation of carotenoids during chromoplast differentiation is C_2H_4 dependent in this tissue.

In conjunction with transcriptome analysis, HPLC was used for carotenoid profiling with wild-type and *Nr* fruit. These HPLC data show that C_2H_4, via the Nr^+ receptor, has two effects on the accumulation of carotenoid metabolites in developing tomato pericarp. Concomitant with fruit ripening, C_2H_4 (1) causes a dramatic increase of carbon flux into this 2° metabolic pathway and (2) alters the kinetics of the pathway such that Lyc accumulates preferentially over β-Car. The shift from the accumulation of β-Car to Lyc is thought to be due to a decrease in the accumulation of transcripts that encode two Lyc cyclase activities (Pecker et al., 1996; Ronen et al., 2000). If this leads to reduced Lyc cyclase activity, it would reduce the conversion of Lyc to α- and β-Car and thereby result in Lyc accumulation. Figure 6 confirms the observation that B^+ transcripts decrease during tomato fruit ripening and extends this observation by demonstrating that B^+ is transcriptionally regulated in a C_2H_4-dependent manner. These findings indicate that fruit pigmentation during tomato ripening is dependent on (1) C_2H_4 and the Nr^+ receptor, (2) a dramatic increase in carotenoid metabolic flux, and (3) a metabolic bottleneck that occurs downstream of Lyc formation and upstream of β-Car formation. This metabolic bottleneck is due, at least in part, to Nr^+-dependent regulation of B^+.

In summary, combined transcriptome/carotenoid analysis indicates that ethylene influences multiple steps in carotenoid synthesis impacting net and relative accumulation of these compounds. Combined genotype, transcriptome, and targeted metabolite analysis has helped define the ethylene-regulated transcriptome of tomato fruit and added to our knowledge of the role of ethylene in both carotenoid and ethylene accumulation in maturing fruit. In order to facilitate utilization of the wild-type and *Nr* fruit development profiles by other researchers, all of the raw and analyzed data presented here have been deposited at the Tomato Expression Database (http://ted.bti.cornell.edu/), which includes tools for viewing and querying the data described herein.

METHODS

Experimental Design

Normal tomato plants (*Solanum lycopersicum* cv Ailsa Craig and cv Pearson), a homozygous isogenic line (cv Pearson), and a line nearly isogenic for the *Nr* mutation (cv Ailsa Craig, backcross parent) were cultivated using standard greenhouse practices (26°C and 12-h supplemental lighting, followed by 12 h at 20°C). To collect stages prior to ripening, fruit were tagged 7 DAP and harvested at one of five time points: 7, 17, 27, 39 (mature green), or 41 DAP (one day prior to breaker stage). The 7 DAP plants have been shown to consistently yield 1-cm fruit for cultivar Ailsa Craig (Giovannoni et al., 1989). We verified that this definition held in our current conditions prior to initiating fruit harvest. Time from 1 cm to ripening can vary by several days depending on season and growth conditions but typically remains consistent within 1 d for a given

crop grown under the same conditions. For this study, we determined 42 days to breaker and confirmed it at the time of fruit collection. Visual inspection was used to cull obvious variants (typically, parthenocarpic fruit), though it should be noted that as a result of our method of collection, the B-1 stage is most likely to be contaminated with tissues that are less mature than targeted (the infrequent fruit breaking at 41 d were removed from the study). To collect ripening stages, fruit were tagged at the breaker stage (B, which typically occurred 42 DAP in these growing conditions) and harvested at one of five time points: B, or, 1, 5, 10, and 15 d later (B, B+1, B+5, B+10, and B+15, respectively). Breaker stage was defined by the first signs of carotenoid accumulation on the external surface of fruit. To maximize developmental synchrony, harvested fruit from each staged were visually inspected externally and internally (e.g., size, shape, pigmentation, seed development, and development of locular jelly), and only fruit (the vast majority) appearing developmentally equivalent were kept for analysis. After tissue selection, outer pericarp was combined in stage-specific pools (15 to 30 fruit), frozen in N_2, and stored at −80°C.

A direct-sequential design was employed for time-course analyses (Yang and Speed, 2002) and replication accomplished via a triple dye-swap design (Kerr et al., 2002; Alba et al., 2004). To identify genes influenced by the *Nr* lesion in ripening pericarp, microarray analysis was also conducted using RNA from the Pearson fruit at B, B+3, and B+10. Here, six biological replicates were conducted for each of three stage comparisons (i.e., WT-B × *Nr*-B, WT-B+3 × *Nr*-B+3, and WT-B+10 × *Nr*-B+10). Dye bias was minimized by conducting half of the replicate hybridizations with wild-type cDNAs labeled with Cy3 and half of the replicate hybridizations with wild-type cDNAs labeled with Cy5. Genes showing differential expression in at least two of the three ripening stages tested were defined as Nr dependent (*t* test, $P < 0.05$, FDR < 0.0025).

RNA Extraction, Microarray Hybridization, and Scanning

Total RNA was extracted from wild-type tissue using a hot phenol method similar to Reymond et al. (2000). cDNAs corresponding to wild-type tissues were prepared via the 3DNA submicro expression array kit (Genisphere) via the Appendix B protocol using 50 μg of RNA. For wild-type fruit, probes were annealed to target cDNAs for 13 to 17 h at 60°C, and arrays were washed thrice for 20 min each: 60°C in 2× SSC/0.2% SDS, room temperature in 2× SSC, and room temperature with 0.2× SSC. Arrays were dried via centrifugation (1 min at 450*g*). 3DNA capture reagent was annealed for 2 h at 60°C, and microarrays were rewashed as above. For *Nr* tissues, cDNA synthesis, labeling, hybridization, washes, and scanning were conducted as by Alba et al. (2004).

Data Acquisition, Filtering, and Processing

Fluorescence data were processed using ImaGene software (version 4.2; BioDiscovery). Low-quality data were identified via five steps: (1) spots were flagged by setting ImaGene's ignored pixels threshold to 69%, the area-to-perimeter threshold to 0.6, and the offset value threshold to 17.5; (2) expression signals with $Sigl_{Mean} < (Bckd_{Mean} + (2 \times Bckd_{StdDev}))$ were deemed indistinguishable from background and flagged (Wang et al., 2001); (3) a q_{com} value was calculated for each feature (Wang et al., 2001), and all expression values with $q_{com} < 0.4$ or $q_{com} < (q_{com}\ mean - (2 \times q_{com}\ SD))$ were flagged; (4) data derived from less than three nonflagged spot signals were flagged; and (5) data derived from features with $CV_{inter\text{-}slide} > 0.4$ were flagged. Signal means were determined without background correction (Rocke and Durbin, 2001), and GeneSpring (version 6.2; Silicon-Genetics) was used to generate log ratios [i.e., $\log(T_{X+1}/T_X)$ or log(*Nr*/wild type)] and for print-tip Lowess normalization (Yang et al., 2002).

Expression profiles were generated when data existed for seven to nine time-course comparisons, and KNN_{impute} (Troyanskaya et al., 2001) was used to approximate missing values. Profiles were obtained by arbitrarily defining expression at 7 DAP as 1.0 and using the series of ratios for each EST to calculate relative expression. Expression profiles were clustered using a self-organizing tree algorithm (Herrero et al., 2001). Transcriptome topology in wild-type fruit was visualized by aggregating expression clusters in a profile-dependent manner and plotting in three-dimensional space (MATLAB, version 6.0; The MathWorks). The *Nr* transcriptome was visualized by placing the *Nr* profiles in an order identical to the wild-type profiles and plotting the data set as above.

Differentially expressed genes were identified using one-way ANOVA (with developmental stage as the test parameter), a per gene variance model, and an FDR (Benjamini and Hochberg, 1995) of 0.05. Parametric tests were conducted without the assumption of equal variance. Candidate regulatory genes were identified using five criteria: (1) they are differentially expressed in wild-type fruit, (2) their expression is altered in *Nr* fruit, (3) they share sequence homology (E-value $\leq 1 \times 10^{-10}$) with known regulatory genes, (4) the ESTs encoding these array features have been verified by sequencing, and (5) pseudoreplicates in the array (i.e., different EST features deriving from the same gene) yield similar expression profiles.

ASC Measurements

ASC determinations were based on ΔA_{265} of after ASC oxidation (Bashor and Dalton, 1999). ASC was extracted from 100 mg of powdered pericarp tissue, and extract supernatant (100 μL) was combined with 895 μL of assay buffer (100 mM KPO_4 and 1 mM EDTA, pH 6.5). Absorbance at 265 nm was determined before and after the addition of ASC oxidase (3.3 units/5 μL), and [ASC] was calculated using an extinction coefficient of 14.1 $mM^{-1} \cdot cm^{-1}$.

Carotenoid Extraction and HPLC

Frozen pericarp (200 mg) was extracted with 600 μL of tetrahydrofuran/methanol and 12.5 mg of Mg carbonate. The homogenate was filtered with a Spin-X filter (Corning) and re-extracted with 550 μL of tetrahydrofuran. Carotenoids were partitioned into 325 μL of petroleum ether using 150 μL of 25% NaCl. The extract was evaporated to near dryness, suspended in methyl *t*-butyl ether:methanol (500:475), and passed through a syringe filter (GE Osmonics) prior to injection onto a C_{30} Carotenoid column (Waters).

HPLC employed a Summit HPLC system and a PDA-100 photodiode array detector (Dionex). The elution gradient consisted of 5 min at 100% methanol, a 20-min ramp to 95% *t*-butyl ether, 5 min at 95% *t*-butyl ether, and a 5-min ramp returning the system to 100% methanol. The column was equilibrated with 100% methanol for 10 min before each run. Spectra were collected at 286, 315, 350, 450, and 471 μm, and pigments were identified via comigration with purified standards and/or by their pigment-specific absorbance spectra. Lyc and β-Car standards were purchased from Sigma-Aldrich. PE and δ-carotene standards were purified in the lab.

Databases for Tomato Functional Genomics

The raw, processed, and supplemental data described here have been deposited in the Tomato Expression Database (http://ted.bti.cornell.edu/) and are available for public access (Fei et al., 2003). Nucleic acid sequence and annotation data pertaining to the TOM1 microarray are available via the SGN (http://sgn.cornell.edu/).

Nomenclature

When possible, gene nomenclature adheres to the conventions of the Tomato Genetics Resource Center, and priority gene names/symbols have been used (http://tgrc.ucdavis.edu/Genes.html). When priority gene names/symbols have not been assigned by the Tomato Genetics

Resource Center, common three-letter abbreviations are used. Gene names/symbols are denoted by italics and proteins names/symbols are denoted by no italics.

Accession Numbers

Sequence data from this article can be found in the SGN database (http://www.sgn.cornell.edu/) under the following accession numbers: 14-3-3 protein (SGN-U213806), *ACO1* (SGN-U212786), *ACS2* (SGN-U216896), *ACS4* (SGN-U220379), *AOBP* (SGN-U218715), *B* (SGN-U221920), *bZIP1* (SGN-U213576), *bZIP61* (SGN-U220645), *CAF1* (SGN-U213840), *CGS* (SGN-U213411), *CrtB-b* (SGN-U221960), *DXS* (SGN-U215419), *E4* (SGN-U213993), *E8* (SGN-U212804), *EXP1* (SGN-U215711), *gh* (SGN-U218630), *MAPKK* (SGN-U215082), *MBF1* (SGN-U217359), *MSR* (SGN-U217937), *Nr* (SGN-U214004), *pds* (SGN-U219813), *PG2A* (SGN-U213213), PP2A regulatory subunit (SGN-U213418), *r* (SGN-U212843), *rin* (SGN-U212614), *SAM1* (SGN-U212824), *TFIIE* β-subunit (SGN-U214207), transducin-like heterotrimeric G-protein (SGN-U219841), and *TS* (SGN-U223897).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1. 869 Genes Detected as Differentially Expressed in Developing Tomato Pericarp.

Supplemental Table 2. Nr Regulates the Development of Mature Green Fruit in Tomato.

Supplemental Table 3. 739 Genes Identified as Differentially Expressed in at Least Two of the Three Stage Comparisons Conducted.

Supplemental Figure 1. Ethylene Regulates Accumulation of Reduced Ascorbate in Pericarp before Tomato Ripening.

Supplemental Figure 2. Verification of Microarray Data.

ACKNOWLEDGMENTS

We thank Jocelyn K.C. Rose, Julia Vrebalov, Cornelius Barry, Shanna Moore, and Mark Wright for advice and comments. Excellent technical assistance was provided by Yang Liu, Xuimei Tang, Ruth White, and Nick Vail. This research was supported by grants from the National Science Foundation (DBI-0116076 and DBI-0211875).

Received July 12, 2005; revised August 15, 2005; accepted September 20, 2005; published October 21, 2005.

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