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RESEARCH ARTICLES

Transcriptome and Metabolite Profiling Show That APETALA2a Is a Major Regulator of Tomato Fruit Ripening [C][W]

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Fruit ripening in tomato (*Solanum lycopersicum*) requires the coordination of both developmental cues as well as the plant hormone ethylene. Although the role of ethylene in mediating climacteric ripening has been established, knowledge regarding the developmental regulators that modulate the involvement of ethylene in tomato fruit ripening is still lacking. Here, we show that the tomato *APETALA*2a (*AP2a*) transcription factor regulates fruit ripening via regulation of ethylene biosynthesis and signaling. RNA interference (RNAi)-mediated repression of *AP2a* resulted in alterations in fruit shape, orange ripe fruits, and altered carotenoid accumulation. Microarray expression analyses of the ripe *AP2* RNAi fruits showed altered expression of genes involved in various metabolic pathways, such as the phenylpropanoid and carotenoid pathways, as well as in hormone synthesis and perception. Genes involved in chromoplast differentiation and other ripening-associated processes were also differentially expressed, but softening and ethylene biosynthesis occurred in the transgenic plants. Ripening regulators *RIPENING-INHIBITOR*, *NON-RIPENING*, and *COLORLESS NON-RIPENING* (*CNR*) function upstream of *AP2a* and positively regulate its expression. In the pericarp of *AP2* RNAi fruits, mRNA levels of *CNR* were elevated, indicating that *AP2a* and *CNR* are part of a negative feedback loop in the regulation of ripening. Moreover, we demonstrated that CNR binds to the promoter of *AP2a* in vitro.

INTRODUCTION

Tomato (*Solanum lycopersicum*) is the primary model for climacteric fruit ripening for a combination of scientific and agricultural reasons. Its fruit plays an important role in the human diet and provides health benefits as a source of vitamins, minerals, and antioxidants (phenolics, folate, lycopene, and β-carotene) (Toor et al., 2005; Carrari and Fernie, 2006; Fraser et al., 2009). Fruit ripening is a complex, genetically programmed process that culminates in dramatic changes in color, texture, flavor, and aroma of the fruit flesh (reviewed in Alexander and Grierson, 2002; Carrari and Fernie, 2006). During tomato fruit ripening, free amino acids increase dramatically and their abundance changes differentially (Boggio et al., 2000; Sorrequieta et al., 2010). Ripening of climacteric fruits is characterized by an autocatalytic increase in respiration and ethylene biosynthesis just prior to the initiation of ripening. Ethylene biosynthesis occurs via a pathway (reviewed in Bleecker and Kende, 2000; Alexander and Grierson, 2002; Lin et al., 2009) involving two key biosynthetic enzymes: 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS), which converts *S*-adenosyl-L-methionine (SAM) into ACC (Sato and Theologis, 1989), and ACC oxidase (ACO), which further converts ACC to ethylene. Unraveling the regulation of these gene activities and the ethylene signaling pathway is important to understanding the processes of ripening, senescence, abscission, and response to stress.

Several genes that regulate tomato ripening through ethylene signal transduction have been identified. These are, among others, encoding two ethylene receptors, *Never-ripe* (Wilkinson et al., 1995; Yen et al., 1995) and *ETHYLENE RESPONSE6* (*ETR6*) (Kevany et al., 2007), and *Green-ripe* (*Gr*), a gene encoding a protein of unknown function (Barry et al., 2005; Barry and Giovannoni, 2006). Ripening-associated transcription factors have been found to regulate, either directly or indirectly, the biosynthesis of ethylene. For example, two transcription factors, the MADS box protein RIPENING-INHIBITOR (RIN) (Vrebalov et al., 2002) and COLORLESS NON-RIPENING (CNR), a SQUAMOSA promoter binding protein (SBP) (Manning et al., 2006),

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were proposed to function early in the transcriptional activation cascade regulating ripening-related processes, including ethylene production and/or signaling. Mutations in both loci cannot be rescued by exogenous application of ethylene. RIN was shown to bind to the promoter of *ACS2* (Ito et al., 2008) as does another MADS box protein, TAGL1, which was subsequently proposed to regulate ripening through regulation of *ACS2* (Itkin et al., 2009; Vrebalov et al., 2009). Another transcription factor, the homeobox protein HB-1, was also recently reported to regulate fruit ripening and *ACO1* expression (Lin et al., 2008). Unraveling both ethylene-dependent and -independent transcriptional networks that regulate fruit ripening is crucial for the understanding of this complex process. Here, we describe a member of another class of genes, an *APETALA2/ETHYLENE RESPONSE FACTOR* (*AP2/ERF*).

AP2 is the founding member of the AP2/ERF superfamily, a family of transcription factors unique to plants. Together with the ethylene-responsive element binding protein and RAV subfamilies, the AP2 subfamily forms the AP/ERF superfamily, which plays important roles in diverse processes, such as development, ethylene response, and pathogen resistance. It is defined by the conserved 68–amino acid DNA binding AP2 domain, first described by Jofuku et al. (1994) for the AP2 protein from *Arabidopsis thaliana*. The AP2 subfamily is distinguished from the ethylene-responsive element binding protein and RAV subfamilies by the occurrence of two AP2 domain copies in each protein, while the proteins in the other two subfamilies have only one copy.

The archetypal *AP2* from *Arabidopsis* (Koornneef et al., 1980; Jofuku et al., 1994) is characterized by mutants that show modifications of the outer two floral organ whorls. In weak *ap2* mutants, sepals are converted to cauline leaf-like structures with stigmatic papillae at their tips, and petals show incomplete conversion into stamens (Bowman et al., 1989). In strong *ap2* mutants, sepals are converted into ovule-bearing carpels and petals are absent (Bowman et al., 1991). *AP2* is also involved in seed development, as shown by alterations in the seed coat epidermis in *ap2* mutants (Jofuku et al., 1994) and regulation of seed size (Jofuku et al., 2005; Ohto et al., 2005). It has been shown that *AP2* and its closest homologs are targets of miR172, which downregulates these target genes by a translational inhibition mechanism rather than by RNA cleavage (Aukerman and Sakai, 2003). The targets of miR172 were shown to regulate flowering time genes from *Arabidopsis* as well. Plants lacking *TARGET OF EAT1* (*TOE1*) and *TOE2* are early flowering, whereas plants overexpressing *TOE1*, *TOE2*, *SCHLAFMUTZE* (*SMZ*), and *SCHNARCHZAPFEN* (*SNZ*) flower late (Aukerman and Sakai, 2003; Schmid et al., 2003; Jung et al., 2007). *AP2* homologs that share similarities in gene structure and function with *AP2* have been isolated from numerous species. The putative *Petunia hybrida* ortholog, *AP2A*, is capable of complementing *ap2* mutants of *Arabidopsis*. In *Petunia*, however, knockout mutations of *AP2A* do not affect floral organ development, suggesting that *AP2* function is redundant in this species (Maes et al., 2001). Similarly, in *Antirrhinum majus*, two close homologs have been identified, *LIPLESS1* (*LIP1*) and (*LIP2*), both of which need to be inactivated to get an *ap2*-like phenotype (Keck et al., 2003).

For tomato, no *ap2*-like mutants have been identified so far. In this study, using a transgenic approach that involves targeted RNA interference (RNAi) repression, we show that the closest tomato homolog of *AP2*, *AP2a*, plays a critical role in fruit ripening. This gene was also recently described as a negative regulator of ripening and of ethylene production (Chung et al., 2010). We found that tomato *AP2*-like genes also function in floral development, similar to *Arabidopsis AP2*, possibly in a redundant manner. In addition, by transcriptome profiling, we identified many direct and indirect target genes of *AP2a*, among which components of the ethylene and auxin signaling pathways and genes involved in the differentiation of chloroplasts into chromoplasts. Significant changes in key primary metabolites and carotenoids were observed as a result of *AP2a* downregulation. Our data show that *AP2a* has positive ripening regulatory functions besides its negative regulatory function in ethylene synthesis. Moreover, we show that the ripening regulators RIN, NON-RIPENING (NOR), and CNR as well as ethylene positively regulate *AP2a* expression in either a direct or indirect manner, while *AP2a* in turn negatively regulates *CNR* expression, thus positioning *AP2a* in a ripening regulatory network that includes a negative feedback loop.

RESULTS

AP2 Homologs in Tomato

To gain insight into the molecular regulation of tomato fruit development, we selected potentially important transcription factor genes for suppression in transgenic tomato using an RNAi strategy. Comparison of EST frequencies in various tomato EST libraries indicated that the *AP2* homolog represented by TC162117 is highly expressed in the pericarp of developing tomato fruit. Publicly available microarray expression data show that the level of mRNA represented by this contig increases steadily during tomato fruit development, to a level at day 48 that was 2.5 times the level at 7 d postanthesis (http://ted.bti.cornell.edu/cgi-bin/array/unigene.cgi, SGN-U213383). This observation and the demonstrated important regulatory role of AP2-like transcription factors in developmental processes indicated that the *AP2* homolog might play an important role in tomato fruit development as well.

We mined the Solanaceae Genomics Network (SGN) tomato Unigene database for other unigenes with homology to *Arabidopsis AP2* and, where necessary, extended cDNA sequences by 5′ and 3′ rapid amplification of cDNA ends to obtain sequences covering the entire open reading frame of each gene. At completion, we had identified five distinct tomato cDNAs encoding *AP2* homologs, including the one represented by the tentative consensus mentioned above. Figure 1A (see Supplemental Data Set 1 online) shows a phylogenetic tree of the encoded proteins together with AP2 homologs of *Arabidopsis*, *Petunia*, and *Antirrhinum*, as far as available. One of these is the previously identified AP2 from tomato represented by Unigenes SGN-U579591, 580201, and 593325, which we here call *AP2a* (*S. lycopersicum AP2a*). For four other genes, full-length cDNA sequences were obtained by 5′ and 3′ rapid amplification of cDNA ends from available Unigene sequences. Two tomato proteins, which are homologous to the previously identified

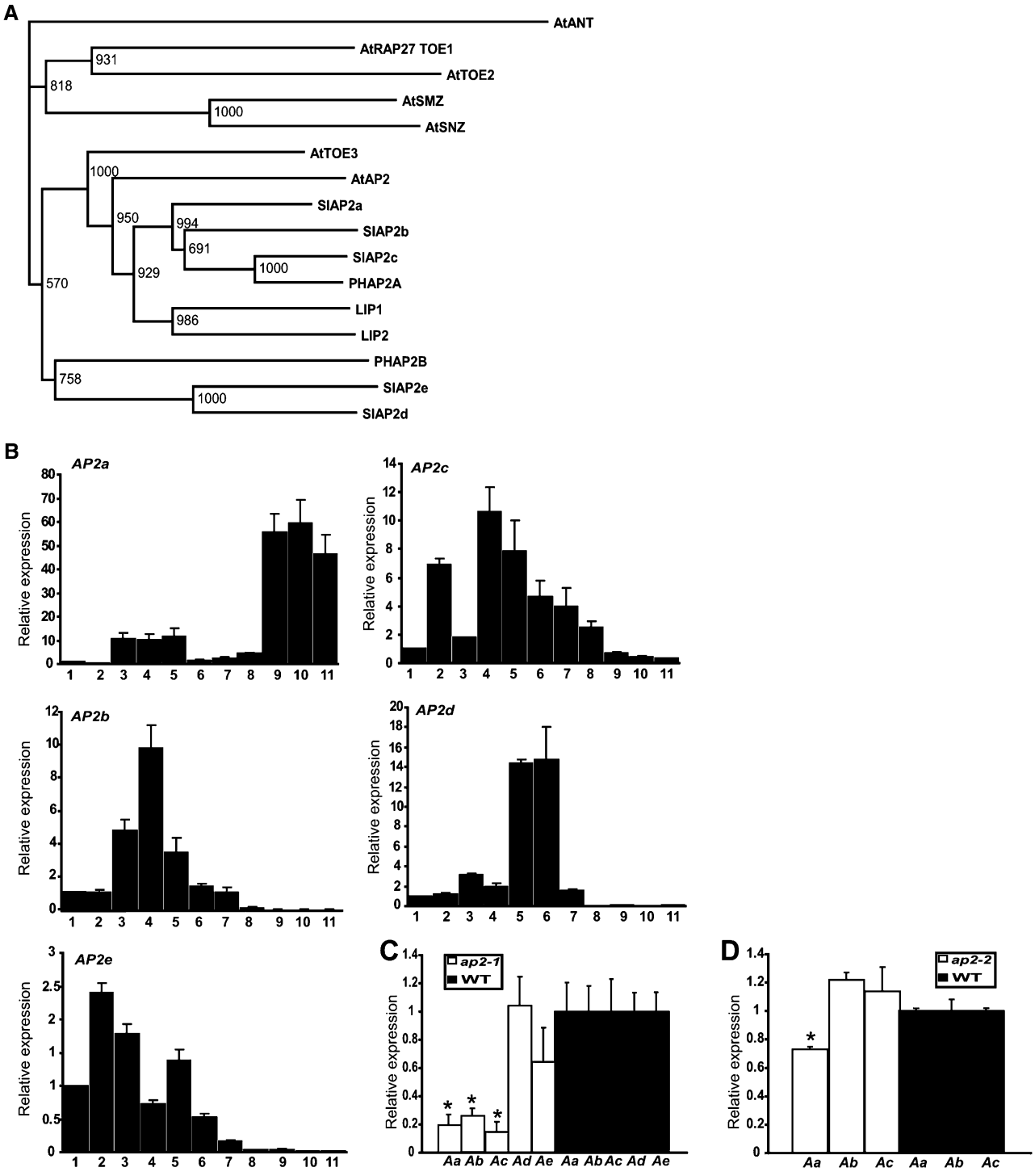

Figure 1. Phylogenetic Analysis of *AP2a* and Its Homologs and Comparison of Their Expression Pattern in Different Wild-Type Tissues and at Various Fruit Developmental Stages.

(A) Protein phylogenetic tree of tomato AP2s and selected homologs indicate that five AP2 paralogs exist in tomato. Numbers at tree nodes indicate bootstrap values from a total of 1000 trials.

(B) Relative expression profiles of *AP2a*, *AP2b*, *AP2c*, *AP2d*, and *AP2e* in different cv Moneymaker tissues obtained by quantitative RT-PCR. 1, Seedlings; 2, leaves; 3, roots; 4, flowers; 5, 5-mm fruits; 6, 1-cm fruits; 7, early green fruits; 8, mature green fruits, 9, breaker; 10, turning; 11-Br+7, 7 d after breaker stage, red fruits. Values represent means of two biological replicates, and vertical bars represent SE of the means.

AP2A from *Petunia*, were designated AP2b (represented by SGN-U580135 and 589866) and AP2c (represented by SGN-U580203 and 579384). These three proteins are identical to AP2a, b, and c, respectively, published recently by Chung et al. (2010). The remaining two tomato AP2-like proteins are more distantly related to *Arabidopsis* AP2, are more similar to the *P. hybrida* AP2B (Maes et al., 2001), and were designated AP2d (represented by SGN-U563871) and AP2e (represented by SGN-U585439 and 585539). A more elaborate phylogenetic three including these five proteins with AP2 subfamily proteins from diverse species is shown in Supplemental Figure 1 online (see Supplemental Data Set 2 online). This analysis firmly places tomato AP2a, b, and c proteins together with *Arabidopsis* AP2 in a subclade of the AP2 subgroup of the AP2 subfamily of transcription factors as defined by Shigyo et al. (2006), indicating that these three proteins are putative tomato orthologs of AP2. Tomato AP2d and AP2e, with *P. hybrida* AP2B, are more similar to *Arabidopsis* TOE1 and TOE2. Several other tomato AP2 subfamily proteins, or fragments thereof, were identified from the SGN Unigene database and were included in the analysis. An alignment of the tomato proteins with *Arabidopsis* AP2, TOE2, and AINTEGUMENTA (ANT) is shown in Supplemental Figure 2A online. The phylogenetic position of AP2d and AP2e is less clear than for the other three proteins (as shown by the low bootstrap values on the major nodes). However, all five proteins belong to the euAP2 lineage, as they all contain a putative miR172 target site and lack the characteristic ANT lineage motifs, such as the 10–amino acid and 1–amino acid insertions in AP2 domain repeats 1 and 2, respectively (Kim and Nam, 2006).

AP2a Expression Increases during Fruit Ripening

To better understand possible functions as well as to identify possible functional redundancy through overlapping expression patterns for the tomato *AP2* genes, we determined, by quantitative RT-PCR (qRT-PCR), the expression levels of the five *AP2*-like genes in tomato seedlings, leaves, roots, and flowers at anthesis, flowers at 2 d postanthesis (Fruit I), and fruit at different developmental stages. All expression levels as related to the expression of the β-actin gene and normalized to the first sample (seedling) are shown in Figure 1B. From this figure it is apparent that of the three genes most related to *Arabidopsis AP2*, *AP2b*, and *AP2c* have similar expression patterns, with highest expression in flowers and some expression in roots, leaves, and early stage fruits, but no significant expression beyond the mature green stage. By contrast, *AP2a* is expressed at a relatively low level in flowers and early fruit stages but is strongly upregulated between the mature green and breaker stages and is highly expressed up to the red ripe stage. *AP2d* and *AP2e* are differentially expressed, with *AP2d* being preferentially expressed in very early fruit development and *AP2e* being expressed particularly in vegetative tissues, but also in flowers. All homologs were expressed in seeds, with *AP2d* and *AP2e* being the highest expressed (see Supplemental Figure 3A online). Thus, the five identified tomato *AP2* paralogs are differentially expressed in vegetative tissues, flowers, and developing fruit. The differential expression of the three closest homologs of *AP2*, with only *AP2a* being expressed in ripening fruit and *AP2b* and *AP2c* both being expressed in flowers, suggests that extensive gene duplication and subsequent subfunctionalization of these *AP2* homologs through differential expression have occurred in the tomato lineage since its divergence from the *Arabidopsis* lineage in evolution. If this is indeed the case, the genes that, like *Arabidopsis AP2*, are predominantly expressed in flowers may function in floral organ identity determination, as *AP2* does, while *AP2a* may have acquired a novel function more specific for fleshy fruit development.

Phenotype of *AP2a* RNAi Lines

We constructed an RNAi vector from a 468-bp cDNA fragment (underlined in Supplemental Figure 2B online) of *AP2a*. The resulting construct, designated *AP2*i-1, contains two copies of a sequence encoding most of the two AP2 domains (see Supplemental Figure 2B online, bold) in an inverted repeat orientation under the transcriptional control of the cauliflower mosaic virus 35S promoter. Transgenic tomato cv Moneymaker plants harboring the *AP2*i-1 construct were generated to examine the effect of the suppressed *AP2a* expression on development.

Out of 12 analyzed diploid transformants, three lines with *AP2*i-1 exhibited severe alterations both in flower development and in fruit development and ripening, and three showed a milder phenotype. Figure 2 and Supplemental Figures 3B to 3E online show representative phenotypes of flowers and fruit of *AP2*i-1 lines. The sepals were greatly increased in size, both in length and in width (see Supplemental Figures 3B and 3C online). Sepals were fused along the margins and curled at the tip, forming a bell shape that completely enclosed the flower, leaving the petals invisible (Figure 2A; see Supplemental Figure 3C online). As the fruits developed, the sepals continued to increase in size (cf. the length and width of sepals in flowers and on fruit for wild-type and transgenic lines in Supplemental Figures 3B and 3C online). Because all five tomato *AP2* paralogs are expressed in flowers, they might be functionally redundant in that organ; therefore, the flower phenotype may be the result of down-regulation of one or more of the other AP2 homologs in addition to *AP2a*. To test this hypothesis, we performed qRT-PCR to

Figure 1. (continued).

(C) Relative expression profiles of Aa, *AP2a*; Ab, *AP2b*; Ac, *AP2c*; Ad, *AP2d*; and Ae, *AP2e* in sepals of *AP2*i-1 transgenic lines or the wild type (WT). Values represent means of two biological replicates, and vertical bars represent SD. The wild-type expression data are normalized to 1. Asterisks indicate P value < 0.05 (*t* test) when comparing data for each measurement between the *AP2*i and wild-type plants.

(D) Relative expression profiles of Aa, *AP2a*; Ab, *AP2b*; and Ac, *AP2c* in the sepals of *AP2*i-2 transgenic lines or the wild type. Values represent means of two biological replicates, and vertical bars represent SD. The wild-type expression data are normalized to 1. Asterisk indicates P value < 0.05 (*t* test) when comparing data for each measurement between the *AP2*i and wild-type plants.

Figure 2. Ripening Effects of Decreased AP2a Expression.

(A) Typical example of the ripening phenotype of *AP2*i-1 RNAi lines. Fully developed fruit (equivalent to Br + 7, 7 d after breaker stage) are orange in color, never achieving the full red color of the wild-type ripe tomatoes at the same stage.

(B) Different stages of fruit ripening: (from left to right) mature green (~40 d postanthesis), turning, pink and red ripe (Br + 7) for wild-type (WT) and *AP2*i-1 RNAi lines. Fruits from independent transformant lines are shown (5, 8, 10, and 11, respectively), which are delayed in color development, never developing full red color.

(C) Ripening wild-type fruits have an even, homogenous color and a smooth, intact surface during the orange and red stages of ripening. The color of the ripening *AP2*i-1 RNAi lines at the Br + 7 stage is not homogenous, with sectors displaying different colors. The fruit does not develop the red color but remains orange, even when the tissue has characteristics of being fully ripe or overripe (i.e., soft and mushy). The surface of the fruit splits open (arrows and in **[A]**).

(D) Compared with the wild type (left), the fruit from *AP2*i-1 lines (right) at the mature green stage has a distorted shape, exhibiting strong indentations resulting in less round fruit. Additionally, the surface of the fruit is bumpy (arrows).

(E) Wild-type (left) and *AP2*i-2-15 RNAi fruits (*ap2-2-15*) at Br + 7, showing ripening defects and uneven pigmentation.

compare the expression of the five *AP2* homologs in *AP2*i-1 and wild-type sepals (Figure 1C). We found that not only *AP2a* but also its closest homologs, *AP2b* and *AP2c*, were downregulated in sepals, while the expression of the other two members of the family, *AP2d* and *AP2e*, was not affected.

Additionally, the morphology of the fruit was altered in some of the *AP2i-1* lines (Figures 2B to 2D). A wild-type fruit (Figure 2D) is round, with a smooth, consistent epidermal surface. Fruits of the *AP2*i-1-8 line, on the other hand, were not round but had large indentations delineating the location of each locular cavity. Another common feature of these fruits was the raised bumpy areas that were present intermittently on the epidermal surface of the fruit (Figure 2D, arrowheads). When the seeds from these *AP2*i-1-8 fruits were closely examined, we found that seed morphology was also affected. In *AP2*i-1 lines, a variety of aberrant seed shapes was observed (see Supplemental Figures 4A to 4E online). Moreover, seed coat morphology, specifically the size and shape of the surface hairs, was strongly affected. In tomato, the seed coat hairs are the cell wall remains of the outer epidermal cells. Their size and shape varies between cultivars and cv Moneymaker has laterally banded hairs as shown in Supplemental Figures 4B and 4E online (Chakrabarti et al., 2003).

AP2a Is Involved in Fruit Ripening

In addition to the alterations observed in the early development of the fruit, *AP2*i-1 lines exhibited distinct alterations in the ripening process (Figure 2). Ripe *AP2*i-1 fruits are shown in Figure 2A. All fruits had ripened, yet never turned from orange to homogenous red. The fruit phenotype for wild-type and *AP2*i-1 lines at various stages of ripening is shown in Figure 2B (several fruits of the same truss, representing different ripening stages, are shown). In the wild type, fruit ripening (Figure 2B, top row, left to right) begins with mature full-sized green fruit. In the next stage, the breaker (Br) stage, a visible color change just begins to occur. By the turning stage, the entire fruit has a consistent orange color. In the final stage (breaker + 7 d) of ripening, the fruit has a deep red color and starts to soften. The differences in ripening between the *AP2*i-1 and wild-type fruits became apparent at the turning stage. At breaker + 7 d, the fruit retained its orange color, even though other aspects of the ripening process, such as tissue softening, had begun. In Figure 2C, fruits from the wild type and two independent *AP2*i lines, *AP2i*-1-8 and *AP2i*-1-10, at turning (top) and breaker + 7 stage (bottom) are shown. Wild-type fruit at the turning stage was a homogenous orange. However, the color of fruit from *AP2*i-1 lines was patchy with sectors of different shades of yellow and orange. At the breaker + 7 d stage, wild-type fruit was homogenous red, whereas *AP2*i-1 lines still displayed sectors of different colors and did not turn red. The internal fruit anatomy at breaker + 7 d is shown in Supplemental Figure 4F online. The inside of wild-type fruit was red throughout. The locular cavities were filled with seeds and jelly, resulting in a juicy appearance. While *AP2*i-1-10 line fruits were more similar to the wild type in texture, fruits from line *AP2*i-1-8 appeared drier and the fruit appeared to be crumbly, with the internal structure collapsing (see Supplemental Figure 4F online). Line *AP2*i-1-8 showed the most severe phenotype. Additionally, the peel of *AP2*i-1-8 fruits was frequently cracked (Figure 2C, arrows). We noted that the *AP2*i-1-8 fruits deteriorated faster than the wild-type fruits (Figure 3A), and this could explain the observed peel cracks in the fruits shown in Figure 2C.

Because the *AP2*i-1 construct targets *AP2a* as well as *AP2b* and *AP2c*, and although the latter two show no expression in ripening fruits, we designed another RNAi fragment named *AP2*i-2 (highlighted in gray in Supplemental Figure 2B online), which excludes the conserved AP2 domains. In agreement with the proposed role of *AP2a* in fruit ripening, fruits from three out of 15 transformant lines containing this construct showed the same ripening defects (Figure 2E), without the sepal or seed phenotypes observed in the *AP2*i-1 lines. This supports the proposed function of *AP2a* in fruit ripening, while flower- and seed-specific functions may be shared with, or are exclusively determined by, *AP2b* and *AP2c*. To confirm this, we performed qRT-PCR to compare the expression of the five *AP2* homologs in *AP2*i-2 and wild-type sepals (Figure 1D). We found that *AP2a* was downregulated in the sepals of *AP2*i-2, but, in contrast with AP2i-1 plants, neither of its closest homologs, *AP2b* or *AP2c*, was downregulated. To confirm downregulation of AP2a in fruits of both types of RNAi lines, we performed protein gel blot analysis using AP2a-specific peptide antiserum. In contrast with the situation in wild-type fruits, AP2a was not detected in the transgenic fruits, in both breaker stage (Br) and in 7 d after breaker stage (Br+7) fruits of *AP2*i-1 or *AP2*i-2 lines (see Supplemental Figure 3F online).

Fruits of line *AP2*i-1-8 were used for more detailed characterization of the ripening-specific function of *AP2a*. In the progeny of this line, the described phenotypes all cosegregated with the transgene construct as determined by PCR.

AP2a Expression Is Stimulated by Ethylene, and Its Repression Resulted in Fruits Defective in Ripening but Producing Higher Levels of Ethylene

To achieve full ripening, tomato fruits require synthesis, perception, and signal transduction of the plant hormone ethylene (Alexander and Grierson, 2002; Kevany et al., 2007). As ripening progresses, the expression of many genes is initiated or upregulated (reviewed in Alexander and Grierson, 2002). We observed that the expression of *AP2a* is increased during ripening. To distinguish if this regulation is ethylene dependent or ethylene independent, we incubated wild-type tomato fruits at the mature green stage with the ethylene precursor ethephon. After 6 h of induction, we observed an 8-fold upregulation of *AP2a* expression (Figure 3B).

To investigate if a high level of ethylene production in the *AP2*i fruits may be responsible for the observed early fruit senescence (Figure 3A), we measured the ethylene production in fruits during ripening. As shown in Figure 3C, *AP2*i-1-8 fruits produced 4- to 6-fold more ethylene at breaker and breaker + 7 stages than wild-type fruits at the same stage. Thus, *AP2a* appears to function as a negative regulator of ethylene synthesis in tomato fruits by a negative feedback loop.

AP2a Downregulation Alters Fruit Carotenoid Levels

The green-to-red color change typical of ripening tomato fruits is largely due to chlorophyll degradation and accumulation of carotenoids, including β-carotene and lycopene (reviewed in Giovannoni, 2001). Lycopene, which confers the red color to ripe tomatoes, accounts for 70 to 90% of the total carotenoids present (Burns et al., 2003; Alba et al., 2005). To examine the underlying causes of the color differences observed between wild-type and *AP2*i ripe fruits in more detail, biochemical analysis of carotenoid (β-carotene, all-trans lycopene, lutein, and their precursor phytoene) levels was performed on wild-type and *AP2a* silenced fruits at a similar developmental stage (Figure 4). The *AP2*i fruits had decreased levels of both phytoene and lycopene. By contrast, an increase in the β-carotene content was found, accounting for the orange color of the ripe *AP2*i fruits. The α-tocopherol content was also slightly decreased in these fruits. Elevated levels of chlorophyll were observed in the *AP2i* fruits compared with the wild-type fruits 7 d after the breaker stage (Figure 4A). No difference in the lutein content was observed between the wild-type and transgenic fruits.

Gene Expression Analysis Identifies Genes Regulated by *AP2a* during Ripening

To identify genes that might be directly or indirectly regulated by *AP2a* in tomato, we compared gene expression between transgenic knockdown plants in the T1 generation, in which the

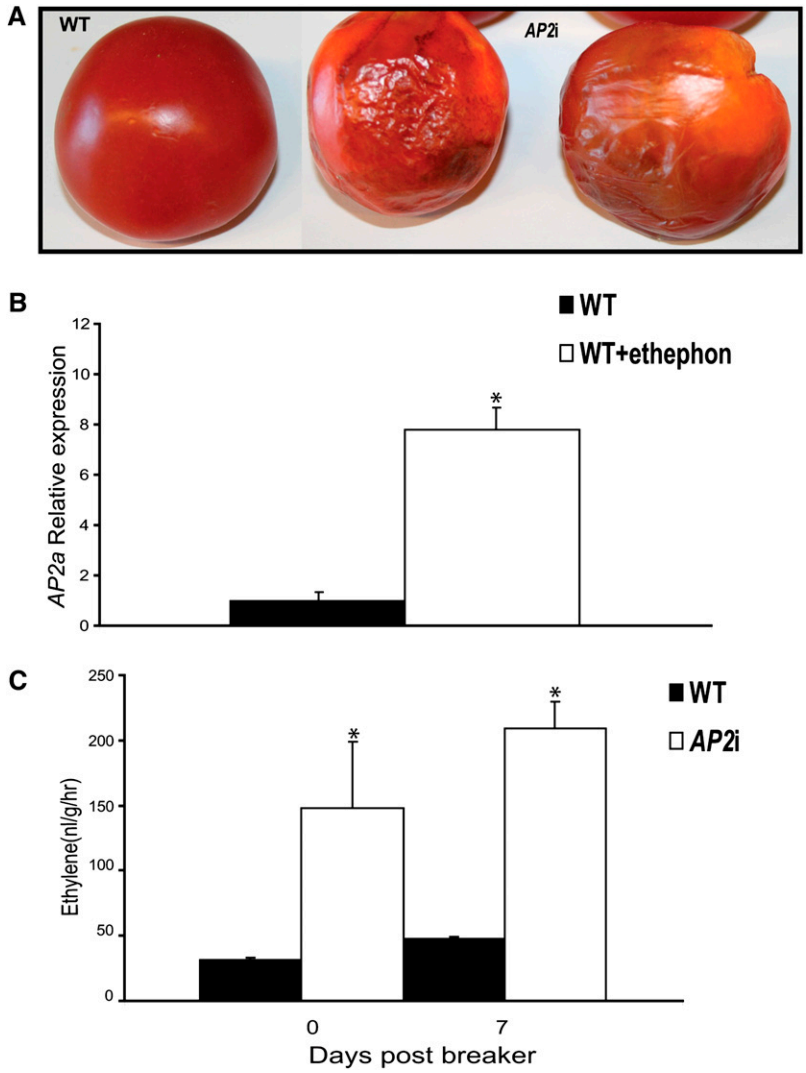

Figure 3. *AP2* RNAi Fruits Senescenced Faster Than the Wild-Type Fruits and Produced More Ethylene.

(A) Comparison of wild-type (WT) and *AP2*i-1 fruits 14 d after harvesting at stage Br + 7 d.

(B) Relative expression profiles of *AP2a* in mature green fruits, treated or not with ethephon. Values represent means of two biological replicates, and vertical bars represent SD. The wild-type expression data are normalized to 1. Asterisks indicate a significant P value < 0.05 (*t* test) when comparing data for each measurement between the treated and nontreated wild-type samples.

(C) Fruits from Br and Br + 7 stages were sealed in airtight vials, and 2.5 mL of gas was sampled after 35 min. Values represent means of at least four fruits, and vertical bars represent SD. Asterisks indicate a significant P value < 0.05 (*t* test).

[See online article for color version of this figure.]

transgene cosegregated correctly with the phenotype, and their wild-type siblings. For this purpose, fruit RNA from three plants derived from transformant line *AP2*i-1-8 and from three plants of wild-type siblings were hybridized to the Syngenta Tomato Affymetrix GeneChip. Analysis of differentially expressed genes revealed 1093 genes with significant differential expression at a P value cutoff of 0.001 (false discovery rate Q-value < 0.002), and genes with a >2-fold difference in expression were identified. After removal of apparent redundancies in the probe set, 527 genes were found to be expressed at significantly lower levels ($2\log \leq -1$), and 523 genes at higher levels ($2\log \geq 1$) in the transgenic fruits. A full list of significantly differentially expressed genes can be found in Supplemental Data Set 3 online. A shortlist of differentially regulated genes, discussed below, is shown in Table 1.

Strikingly, the extreme ends of the spectrum of *AP2a*-regulated genes, as shown in Table 1, are made up predominantly of genes involved in heat shock responses (downregulated in RNAi fruits, 7- to 50-fold) and by genes involved in ethylene synthesis and signal transduction, as well as ethylene-(co-)regulated pathogen and wounding response genes (upregulated in RNAi plants 7- to 58-fold). Although heat shock–responsive genes have not been described in association with fruit ripening, inspection of

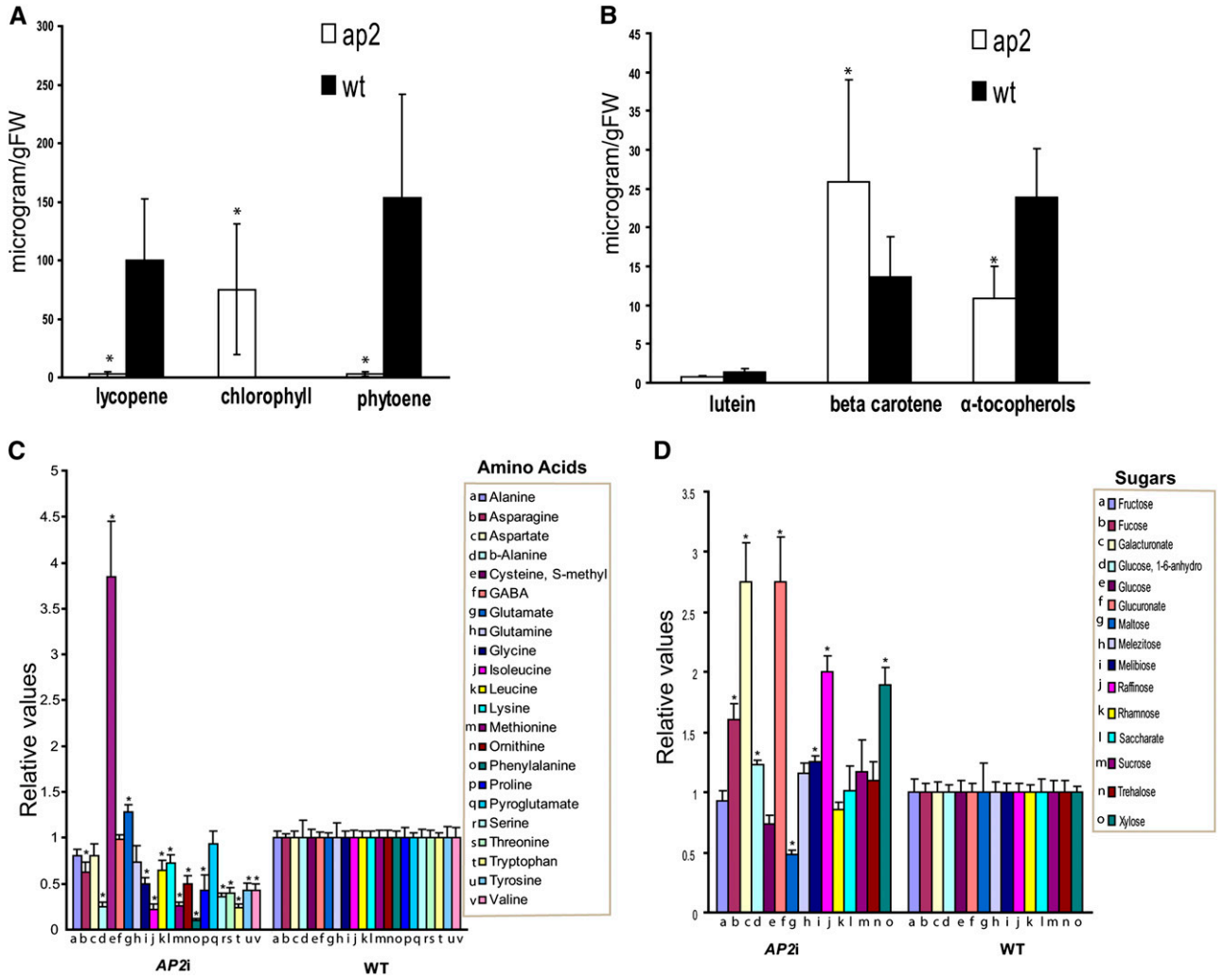

Figure 4. Metabolic Comparison of the *AP2*i-1 and Wild-Type Fruits.

(A) and **(B)** Comparison of the chlorophyll, carotenoid, and α-tocopherol levels (microgram/g fresh weight [FW]) in wild-type (wt) and transgenic *AP2*i-1 tomatoes at stage Br + 7. Biological replicates (11 fruits per sample) were performed in triplicate, and the data are presented as means ± SD. Student's *t* test was used to determine significant differences between wild-type and transgenic fruits $P < 0.01$ (asterisks).

(C) and **(D)** Comparison of the primary metabolites (amino acid and sugar) levels in wild-type and *AP2*i-1 tomatoes (six biological replicates) at stage Br + 7. Wild-type values are normalized to one. Vertical bars represent SE of the means. Asterisks show statistically significant changes according to Student's *t* test ($P < 0.05$).

[See online article for color version of this figure.]

publicly available gene expression data derived from Alba et al. (2005) and Ozaki et al. (2010) shows that many of these are upregulated from the breaker stage onwards, indicating that *AP2a* positively regulates these genes during ripening. Pathogenesis-related proteins that are also ethylene induced in other tissues are not known to be regulated during ripening, and the significant higher expression of these genes in fruits of RNAi plants may represent misregulation of their expression as a result of *AP2a* downregulation, higher ethylene production, or both. Several genes encoding pathogenesis-related proteins were identified as being enriched in the *Cnr* mutant fruit mRNA in a differential cDNA screen against wild-type fruit, suggesting that these genes are also negatively regulated by *CNR* (Eriksson et al., 2004). Several genes that were previously characterized as being ripening induced (Alba et al., 2005) had altered expression levels in *AP2* RNAi fruits, but with opposite effects. *LIPOXYGENASE C* (*LOXC*), *PECTIN METHYLESTERASE 1.9*, *EXPANSIN3* (*EXP3*), and *LOXB* were all downregulated compared with the wild-type fruits. On the other hand, *MITOGEN-ACTIVATED PROTEIN KINASE1* (*MPKA1*), *MITOGEN-ACTIVATED PROTEIN KINASE KINASE4* (*MKK4*), and *MULTIPROTEIN BRIDGING FACTOR1* (*MBF1*), which are all induced during ripening as well, were upregulated in the RNAi fruits compared with wild-type fruits (Table 1). These observations suggest that *AP2a* may

Table 1. Gene Expression Changes for Selected Ripening-Related Genes in *AP2* RNAi Ripe (Breaker +7 d) Fruits

SGN Unigene	Possible Function	Fold Change RNAi/Wild Type	Annotation	Reference
SGN-U581293	–	−49	HSP21/pTOM11 (chloroplast)	Alba et al. (2005)
SGN-U578203	–	−27	Putative HSP20	Alba et al. (2005)
SGN-U566498	Brassinosteroid synthesis	−25	DWARF	Ozaki et al. (2010)
SGN-U578017	Metabolic intermediate biosynthesis	−24	HMG-CoA reductase 2	Rodriguez-Concepcion and Gruissem (1999)
SGN-U572041	Lipid metabolism; oxylipin biosynthesis	−14	LOXC	Alba et al. (2005); Eriksson et al. (2004)
SGN-U584210	–	−11	HSP70 (chloroplast)	Alba et al. (2005)
SGN-U579266	–	−6.7	Putative heat shock protein	Alba et al. (2005)
SGN-U573533	Hormone metabolism, auxin induced	−6.7	GH3.3 (indole-3-acetic acid amido synthetase)	Ozaki et al. (2010)
SGN-U572734	Ripening chlorophyll degradation	−6.6	SGR1 (stay green protein)	Barry et al. (2008)
SGN-U563074	Cell wall metabolism	−6.3	PME1.9 (pectin methylesterase)	Eriksson et al. (2004)
SGN-U581241	Brassinosteroid synthesis	−5.2	DWF1 (DIMINUTO)	Ozaki et al. (2010)
SGN-U576599	Cell wall metabolism	−4.9	EXP3	Eriksson et al. (2004)
SGN-U584987	Carotenoid biosynthesis	−3.5	CrtISO (carotenoid isomerase)	Isaacson et al. (2002)
SGN-U582628	Chlorophyll degradation	−3.3	ACD2; RCCR (red chlorophyll catabolite reductase)	–
SGN-U578028	Lipid metabolism	−3.1	LOXB	Eriksson et al. (2004)
SGN-U568611	Carotenoid biosynthesis	−2.8	CrtR-b2	Bramley (2002)
SGN-U580564	Auxin metabolism	−2.8	SAUR family (auxin responsive)	–
SGN-U569639	Auxin responses	−2.6	ARF4/DR12	Jones et al. (2002)
SGN-U582562	Carotenoid desaturation	−2.4	PTOX (plastid terminal oxidase)	Josse et al. (2000)
SGN-U565008	–	−2.3	FtsH-like protein Pftf	–
SGN-U576134	–	−2.2	ORANGE, DNAJ-like protein	–
SGN-U593894	Carotenoid biosynthesis	−2.3	PDS1 (phytoene desaturase)	–
SGN-U583842	Ethylene biosynthesis	2.2	ACS6	Barry et al. (2000)
SGN-U580893	Ethylene biosynthesis	2.3	SAM3	–
SGN-U576603	Pathogen and insect response	2.5	MPKA1	–
SGN-U571201	Cell wall metabolism	2.6	MANS2	–
SGN-U577086	Ethylene signaling	3.0	ETR5	Kevany et al. (2007)
SGN-U580563	Cell wall metabolism	3.2	Polygalacturonase	–
SGN-U564043	Protein phosphorylation	3.9	MKK4	Alba et al. (2005)
SGN-U569421	Carotenoid biosynthesis	3.9	ZEP1 (zeaxanthin epoxidase)	Thompson et al. (2000)
SGN-U579176	Cell wall metabolism	4.0	MAN3	–
SGN-U563352	Ethylene signaling	4.2	MBF1b/ER24	Zegzouti et al. (1999)
SGN-U571269	Auxin signaling	5.3	MONOPTEROS-like protein	–
?	Auxin signaling	5.4	GH3/ IAA synthetase	–
SGN-U572472	Auxin metabolism	6.6	Putative SAUR family protein	–
SGN-U578110	Protein degradation	6.8	EBF1	Guo and Ecker (2003)
SGN-U579235	Biotic stress response	7.0	PR-P2	–
SGN-U579104	Ethylene biosynthesis	7.1	LeACO4	Nakatsuka et al. (1998)
SGN-U569953	Amino acids metabolism	8.2	BCAT1	Maloney et al. (2010)
SGN-U581694	Ethylene signaling	9.2	ETR6	Kevany et al. (2007)
SGN-U580736	Flavonoid synthesis	12	Phe ammonia lyase	–
SGN-U565756	Regulation of transcription	12	AIM1 (MYB TF)	Abuqamar et al. (2009)
SGN-U570620	Cell wall metabolism	14	CEL1 (endo-1,4-β-glucanase)	–
SGN-U585765	Ethylene signaling	15	EIL2	Tieman et al. (2001)
SGN-U578033	Biotic stress response	79	PepEST (esterase, pathogen, wounding and jasmonate-induced)	–
SGN-U580143	Defense response	85	PR6/PR1b1	–

have activating and suppressing effects on genes that are normally induced during ripening.

During tomato fruit development, significant changes occur in the cell wall components and in the expression of polysaccharide-degrading enzymes (reviewed in Carrari and Fernie, 2006). We found several of the genes encoding these enzymes to be differentially regulated in the *AP2* RNAi fruits. A polygalacturonase gene (*PG*) is significantly upregulated compared with the wild type, as are genes encoding endo-1,3-1,4-β-D-gluconase (*CEL1*), mannosidase (*MAN3*), and mannan synthase (*MANS2*).

A *PECTIN METHYLESTERASE1* (*PE1*) gene and the gene encoding expansin precursor EXP3, both found to be downregulated in the *Cnr* mutant, were also downregulated in *AP2* RNAi fruits (Table 1). These data suggest that AP2a and CNR regulate a number of cell wall–related genes in a partially overlapping manner.

In agreement with the observed higher ethylene production in *AP2* RNAi plants (Figure 3C), we found genes involved in ethylene synthesis, specifically *SAM3*, *ACO4*, and *ACS6*, to be upregulated in the transgenic fruits, suggesting that, during ripening in wild-type fruits, *AP2a* represses ethylene production via these genes. Genes involved in ethylene signal transduction that were found to be significantly upregulated in the transgenic fruits are *EIN3-LIKE2* (*EIL2*), *ETR5*, *ETR6*, and *EIN3 BINDING F-BOX1* (*EBF1*).

In *AP2i* plants, several carotenoid biosynthesis genes, *PHYTOENE DESATURASE* (*PDS*), *CAROTENE-β-HYDROXYLASE* (*CtR-b2*), and *CAROTENE ISOMERASE* (*CtrISO*), were significantly downregulated (Table 1). Expression of the other carotenoid synthesis genes was detected but not significantly altered. Additionally, we found *ZEP1*, which encodes zeaxanthin epoxidase and is involved in abscisic acid synthesis, to be upregulated (4-fold) in the transgenic fruits. These results suggest that *AP2a* positively regulates carotenoid synthesis during ripening through activation of specific genes in the biosynthetic pathway.

The expression of several key transcripts related to biosynthetic genes involved in phenylpropanoid and flavonoid formation was also changed in the *AP2* RNAi fruits (Table 1; see Supplemental Data Set 3 online).

We found several genes that are possibly involved in hormone synthesis to be downregulated in the transgenic fruits. Among these are two genes that are involved in brassinosteroid synthesis: the *D* (dwarf) gene and a *DWARF1*/*DIMINUTO*-like gene (*DWF1*). These results suggest that *AP2a* positively regulates brassinosteroid synthesis during ripening. Furthermore, we observed that the auxin response factor *ARF4* gene was downregulated. Upregulation of a *MONOPTEROS*/*ARF5* homolog and of two homologs of early auxin-responsive genes, *gh3*, and a SAUR family protein homolog and downregulation of another *gh3* homolog and SAUR family homolog suggest that auxin responses are perturbed in these plants.

Finally, we found the expression of a number of genes implicated in chromoplast differentiation to be significantly downregulated. These were the *STAY GREEN PROTEIN1* (*SGR1*) gene, *RCCR* (encoding red chlorophyll catabolite reductase), a tomato homolog of the cauliflower (*Brassica oleracea*) *ORANGE* gene (*OR*), and a homolog of a target of the latter, a tomato homolog of the pepper *PFTF* gene.

AP2a Silenced Fruits Show Increased Accumulation of Sugars and Putrescine and Reduced Free Amino Acid Levels

To identify differentially regulated metabolic pathways in *AP2*i-1-8 plants, we analyzed our microarray data using the Plant MetGenMAP database (Joung et al., 2009). Among the pathways identified, we found an overrepresentation of primary metabolic processes (see Supplemental Table 1 online). Correspondingly, gas chromatography–mass spectrometry (GC-MS) profiles (Lisec et al., 2006) revealed that the *AP2a* silenced fruits displayed substantial changes in the level of primary metabolites (Figures 4C and 4D; see Supplemental Table 2 online). To visually compare alterations in sectors of metabolism and interactions between metabolite levels in the *AP2*i fruits and the wild type, the fold changes in metabolite levels were painted onto biochemical pathway displays (see Supplemental Figure 5 online). The majority of the amino acids (15 out of 22) were present at significantly lower levels in the *AP2*i fruits than in the wild type, and the most dramatic reductions were in β-Ala, Ile, Met, Phe, and Trp. The observed reduced levels of Ile and Leu (branched-chain amino acids) are in agreement with the higher expression of *BRANCHED-CHAIN AMINOTRANSFERASE1* (*BCAT1*; 8.6-fold), which was shown to be involved in the degradation of these two amino acids (Maloney et al., 2010). Similarly, nine of the 15 organic acids measured displayed significant changes. However, these were predominantly increased in the *AP2*i line, with the exception of the tricarboxylic acid cycle intermediates citrate, fumarate and succinate, which were decreased. The increase in benzoate, dehydroascorbate, glycerate, glycolate, nicotinate, and nonanate was relatively mild. The fatty acids dodeconate and octodeconate were also present at mildly, yet significantly, higher levels in the *AP2*i line. Of the 15 sugars we detected, seven (Fuc, galacturonate, glucose-1,6-anhydroglucuronate, melibiose, raffinose, and Xyl) were present at higher levels in the *AP2*i line, whereas maltose was present at lower levels. With the exception of maltose and raffinose, these sugars and sugar derivatives are generally associated with cell wall or ascorbate metabolism. It is interesting to note that the content of the major sugars of the fruit were consistent between genotypes. The content of sugar alcohols in the genotypes was somewhat variable, with an increase in glycerol content but a decrease in inositol in the *AP2*i line. By contrast, inositol 3-phosphate levels were higher in the *AP2*i line, whereas all other sugar phosphates were invariant. Of the six miscellaneous compounds we measured, five were present at significantly different levels between the genotypes, with gluconate-1,5-lactone, phosphorate, putrescine, 3-hydroxy-pyridine, and 3-caffeoyl quinate being elevated in the *AP2*i line. Putrescine was elevated to levels that were 12-fold higher than those observed in the wild type.

Regulatory Interactions between *AP2a* and Other Ripening Regulators

Several transcription factors are positive regulators of tomato fruit ripening. Although *AP2*i fruits failed to complete ripening, they did soften in a similar way as wild-type fruits, in contrast with, for example, *Cnr* mutant fruits (Thompson et al., 1999). The *Cnr* tomato mutant has a colorless, nonripening phenotype and mealy pericarp (Eriksson et al., 2004; Manning et al., 2006). *CNR* and other ripening regulators, like *RIN* and *TAGL1*, were found to have ethylene-dependent and ethylene-independent functions (Giovannoni, 2007; Itkin et al., 2009; Vrebalov et al., 2009). We investigated *AP2a* expression in *nor* (Tigchelaar et al., 1973), *rin*, *Gr*, and *Cnr* mutant backgrounds (Figure 5A). The expression of *AP2a* appeared to be reduced in all four mutants. Our results suggest that, although *AP2a* appears at first sight to be a negative regulator of ripening through inhibition of ethylene

production, it is itself positively regulated by essential regulators of ripening, like CNR, RIN, and NOR (Barry et al., 2005; Giovannoni, 2007; Cantu et al., 2009), and its expression is ethylene dependent, as shown by its downregulation in the *Gr* mutant. Next, to test whether the CNR protein could bind directly to the *AP2a* promoter, we expressed the full-length CNR protein tagged with glutathione *S*-transferase (GST) (GST-CNR) in *Escherichia coli* (Figure 5B). Two *AP2a* promoter regions were selected, one containing the predicted (GTAC) core binding motif of the plant-specific SBP domain (Birkenbihl et al., 2005) and the other one not containing the binding site, to be used as a negative control. To confirm that CNR binds specifically to this core element, we performed an electrophoretic mobility shift assay using the two selected promoter elements (Figure 5B). Our data showed that CNR can bind specifically to the promoter element of *AP2a* in vitro and positively regulates its expression in vivo.

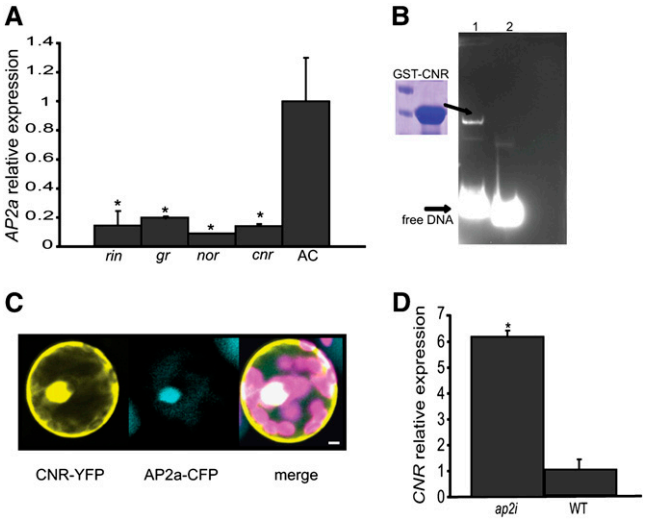

Figure 5. Relative Expression of *AP2a* in Fruits from Different Ripening-Defective Mutant Backgrounds.

CNR protein binds directly to the promoter element of *AP2a*, and the two proteins colocalized in the nucleus of plant cells.

(A) Relative expression profile of *AP2a* in wild-type cv WT-AC (Ailsa Craig), *nor*, *rin*, *Gr*, and *Cnr* fruits at Br +7 d, obtained by qRT-PCR. Values represent means of two biological replicates, and vertical bars represent SD. The wild-type expression data are normalized to 1. Asterisks indicate P value < 0.05 (*t* test).

(B) SDS gel (left) showing GST-purified CNR protein used for the gel retardation assay. Main panel, gel retardation experiments with biotinylated DNA probes and GST-CNR purified protein. 1, *AP2a* promoter element containing the SBP true binding site; 2, *AP2a* promoter element, which does not contain the conserved SBP binding motif, used as a negative control.

(C) Confocal scanning laser microscopy images of an *Arabidopsis* protoplast cotransfected with both *CNR-YFP* and *AP2a-CFP* constructs. Bar = 5 μm.

(D) Upregulation of *CNR* in the pericarp of Br stage of the *AP2*i fruit compared with the wild-type cv Moneymaker (WT). The relative expression levels were obtained by qRT-PCR. Values represent the means of two biological replicates, and vertical bars represent SD.

[See online article for color version of this figure.]

Furthermore, CNR and AP2a were found to be colocalized in the nucleus of transfected *Arabidopsis* protoplast cells (Figure 5C).

The ripening regulatory transcription factors NOR, CNR, and RIN activate the expression of the *AP2a* transcription factor, which in turn acts partially as a negative regulator of ripening through modulation of ethylene signaling. Our microarray analysis was performed with RNA from *AP2*i whole fruits and yielded only a few transcription factors that were significantly regulated (Table 1; see Supplemental Data Set 3 online). Therefore, we decided to investigate in greater detail the expression of *CNR*, *RIN*, and *TAGL1* specifically in the pericarp of the *AP2i* fruits by qRT-PCR. Although no significant change in the expression of *RIN* or *TAGL1* was observed (see Supplemental Figure 6 online), *CNR* mRNA levels were significantly higher in the pericarp of *AP2i* fruits (Figure 5D). Taking into account our previous observation that *AP2a* is positively regulated by *CNR*, these data suggest that *AP2* in the pericarp of wild-type fruits suppresses the expression of *CNR* by a negative feedback loop.

DISCUSSION

In this article, we characterized a putative tomato ortholog of *AP2*, here named *AP2a*. We studied the function of the AP2a transcription factor by analyzing transgenic plants silenced for the *AP2a* gene. A phenotype in tomato fruit ripening was observed, which is similar to that described by Chung et al. (2010). The function of the other tomato *AP2* homologs was apparent from the use of another RNAi construct that targets the conserved AP2 domains, which resulted in additional floral phenotypes. In agreement with Chung et al. (2010), we found that *AP2b* and *AP2c* are not significantly expressed in ripening fruit, thus showing that, also in plants containing the less specific *AP2*i-1 construct, the fruit ripening phenotype is due to downregulation of *AP2a*. Transcriptomic and metabolic analysis of the *AP2*i-silenced fruits described in this study indicate that the AP2a transcription factor plays important roles during tomato fruit development in ripening-related metabolism, in ethylene biosynthesis and signaling pathways, and in the differentiation of chromoplasts. Although, using qRT-PCR, we found a decrease in the mRNA of target gene(s) in the sepals of the RNAi lines, we did not detect such a decrease in ripe fruits, where *AP2a* expression is highest. This observation was supported by the microarray analysis, which also did not detect a significant decrease in *AP2a* mRNA in transgenic fruits. Nonetheless, at the protein level, AP2a was downregulated in fruits of both types of RNAi plants. These results point to a relatively inefficient cleavage of the mRNA and, hence, a relatively inefficient degradation of the endogenous mRNA. In *Arabidopsis*, AP2 protein expression is regulated by a microRNA, miR172, which acts as a translational repressor (Aukerman and Sakai, 2003; Chen, 2004). It was shown that cleavage of the endogenous *AP2* mRNA occurs also but is normally not detectable because of feedback regulation of AP2 transcription by the AP2 protein (Schwab et al., 2005). It was suggested that although perfect or near-perfect complementarity between miRNA and target sequence is usually thought to cause the miRNA to enter the RNAi pathway (Rhoades et al., 2002), and cleavage products of the target mRNA can be

detected by PCR in many cases, it is possible that translational inhibition may be more common than appreciated so far (Aukerman and Sakai, 2003).

AP2b, *AP2c*, or Both, Possibly with *AP2a*, Function in Floral Organ Development

The flower phenotype of the tomato *AP2*i-1 lines is reminiscent of some of the aspects of weaker *ap2* phenotypes in *Arabidopsis* as well as of that in *Antirrhinum lip1 lip2* double mutants (Bowman et al., 1989; Keck et al., 2003). The *Arabidopsis* A-class genes, *APETALA1* (*AP1*) and *AP2*, confer sepal identity in the first floral whorl. While *MADS-MC* (Macrocalyx) is a likely ortholog of *AP1* (Vrebalov et al., 2002), a gene or genes with an A-class function similar to that of *AP2* has not yet been found in tomato. Here, we found that sepals of *AP2*i-1 lines are elongated and show distinct leaf-like characteristics, such as a thinner blade in which venation can be easily observed. However, this sepal phenotype was not observed in the transgenic plants expressing the *AP2*i-2 construct for silencing, suggesting that there may be additional tomato *AP2* genes that are either solely responsible for this sepal phenotype or functionally redundant with each other or with *AP2a* for the floral development function, similar to the redundancy observed in *Antirrhinum AP2* genes. We identified five homologs of the *Arabidopsis AP2* gene in tomato and showed that the observed sepal phenotype in the *AP2*i-1 lines is correlated with downregulation of *AP2a* and its closest homologs, *AP2b* and *AP2c*. Unlike in strong *Arabidopsis ap2* mutants (Bowman et al., 1989, 1991) and in *Antirrhinum lip1 lip2* mutants (Keck et al., 2003), we observed no changes in the other floral whorls. However, similar to *Arabidopsis ap2* mutants, suppression of tomato *AP2* homologs also affected seed shape and seed coat morphology. The *AP2a* suppression could cause defects in the development of the seed testa, specifically in the outer epidermal layer as in *Arabidopsis ap2* mutants (Jofuku et al., 1994; Leon-Kloosterziel et al., 1994), and may well explain the aberrant seed hair morphology that we observed in the *AP2*i-1 lines.

SlAP2a Functions in Fruit Development

Our data demonstrate that *AP2a* plays a role in fruit development. The effect of *AP2* suppression on early fruit development is a bumpy appearance of the green fruits, which is conceivably caused by a defect in the normal or coordinated expansion of the pericarp. Ripening fruits had a number of anatomical differences from wild-type fruits, such as, to a varying degree, a smaller volume of jelly, a dry and crumbly appearance (observed in one line only) of the pericarp, and cracking of the skin, indicating a defect in the expansion or elasticity of the epidermis. Differences in the ripening process were apparent from the differences in color development between wild-type and *AP2*i lines. Orange fruits of tomato *AP2*i lines had a blotchy appearance with light colored or white sectors and failed to turn completely red. Biochemical analysis of the major colored compounds of the tomato fruit, the carotenoids, indicated that *AP2*i fruits are affected in phytoene and lycopene production, explaining their failure to turn fully red. The reduction in color development correlates with the reduced expression of *PDS*, *PTOX*, and *CRTISO* in the *AP2*i fruits, since they encode proteins involved in the biosynthesis of the red all-trans lycopene (for review, see Bramley, 2002). *PDS*, *PTOX*, and *CRTISO* expression in tomato fruits increases dramatically during tomato fruit ripening (Josse et al., 2000; Isaacson et al., 2002), as does expression of *AP2a*. Although reduction of the expression of *CrtR-b2* was also detected, no difference in lutein accumulation was observed in the *AP2*i transgenic fruits (CrtR-b converts α-carotene to lutein together with CrtR-e; Bramley, 2002). Two genes involved in the biosynthesis of flavonoids, *FLAVONOL SYNTHASE* (*FLS*) and *CHALCONE SYNTHASE2* (*CHS2*) (Table 1), were also found to be downregulated in *AP2i* fruits, and the accumulation of tocopherols was reduced when compared with the wild-type fruits.

Carotenoids, flavonoids, and tocopherols are all bioactive compounds with potent antioxidant properties. Carotenoids can be further metabolized to abscisic acid in plants and to vitamin A in animal cells (reviewed in Cunningham and Gantt, 1998). In this study, we demonstrated that *AP2a* positively regulates the synthesis of several carotenoids and of tocopherol during tomato fruit ripening. One of the enzymes dramatically downregulated (24-fold) in the *AP2*i fruits is 3-hydroxy-3methylglutaryl-CoA (HMGR) reductase 2 (Table 1). The HMGR reaction makes mevalonate, a necessary component in the synthesis of all isoprene-containing compounds, such as sterols, carotenoids, phytoalexins, and other growth hormones (Rodriguez-Concepcion and Gruissem, 1999). The expression of *HMG2* was shown to increase strongly during ripening and in parallel with the accumulation of lycopene (Rodriguez-Concepcion and Gruissem, 1999).

AP2a Regulates the Expression of Genes Involved in Chromoplast Differentiation

Differentiation of chloroplasts to chromoplasts is an integral part of tomato fruit ripening, and chromoplasts are the site of lycopene production and accumulation. Chromoplast differentiation involves extensive changes in plastid ultrastructure, accompanied by similarly extensive changes in gene expression (for review, see Egea et al., 2010). A large number of genes that change expression during chromoplast differentiation or have a function in the process have been identified through transcriptome profiling and by natural mutations in genes affected in the process. Several of these are downregulated in our *AP2* RNAi plants, indicating that AP2a may be a major regulator of the process. These genes are *SGR1*, as well as a gene encoding a predicted putative RCCR (red chlorophyll catabolite reductase), which is involved in chlorophyll degradation (Barry et al., 2008), and a tomato homolog of the cauliflower *OR* gene, which encodes a plastidic DnaJ-like protein. The mutation *or* in cauliflower induces the production of chromoplasts in the normally white cauliflower curd, causing it to turn orange due to the accumulation of carotenoids (Lu et al., 2006). In *AP2*i fruits, a tomato homolog of the pepper (*Capsicum annuum*) *Pftf* gene, which encodes an FtsH-like protein, the homolog in cauliflower of which is positively regulated by *Or* (Lu et al., 2006), is downregulated. Apart from the *Or* gene homolog, many other heat shock protein-encoding genes are strongly downregulated in the

*AP2*i plants, several of which were identified as being plastid localized. DnaJ-like cochaperones interact with chaperones, such as Hsp70 (which is also downregulated in *AP2*i plants) and are involved in functions such as protein folding, assembly/disassembly, and translocation, which may be required in higher amounts during chromoplast differentiation. The other chloroplast-localized heat shock protein homologs may have similar functions. As downregulation of *AP2a* likely interferes with this process, the downregulation of the genes encoding the chloroplast-localized heat shock protein homologs in *AP2*i plants would follow from this.

AP2a Is a Negative Regulator of Ethylene Production

Climacteric fruits such as tomato are distinguished from nonclimacteric fruits by their increased respiration and ethylene biosynthesis rates during ripening. Ethylene is formed from Met via *S*-adenosyl-L-methionine (AdoMet) and the cyclic nonprotein amino acid ACC. ACC is formed from AdoMet by the action of ACS and the conversion of ACC to ethylene is performed by ACO (Kende, 1989). Biochemical evidence suggests that ethylene production may be influenced or regulated by interactions between its biosynthesis and other metabolic pathways (reviewed in Carrari and Fernie, 2006). For example *S*-adenosylmethionine is the substrate for both the polyamine pathways and ethylene. SAM, one of the enzymes in the ethylene biosynthesis pathway that is also required for the biosynthesis of polyamines, was found to be upregulated in the *AP2*i transgenic fruits. High levels of putrescine, which together with *S*-adenosylmethionine form spermidine in the polyamine biosynthetic pathway, were observed. The lower levels of Orn (a precursor of putrescine biosynthesis) found in the *AP2*i fruits correlate with higher levels of putrescine. The lower levels of Met measured in the *AP2a* silenced fruits could be attributed to the higher levels of ethylene production, since these fruits produced up to 5-fold more ethylene than the wild-type fruits. This observation suggests that the AP2a transcription factor is a negative regulator of ethylene biosynthesis in ripening fruits. While ethylene action is associated with senescence and ripening, polyamines, on the other hand, have been considered to be senescence inhibitors. Interestingly, in the Sl *AP2*i fruits, both ethylene and putrescine levels are elevated. In general, putrescine is the predominant polyamine during tomato ripening, and its levels are high at the immature-green stage and decline throughout the ripening process (Rastogi and Davies, 1991), which coincides with the accumulation of *AP2a* transcript. Together, our data suggest that *AP2a* is a negative regulator of both ethylene and putrescine synthesis in wild-type fruits. Interestingly, we show that *AP2a* expression is induced by ethylene, suggesting that *AP2a* indirectly regulates ethylene biosynthesis by a negative feedback loop mechanism.

Ethylene biosynthesis is tightly regulated, and two systems of ethylene regulation have been proposed to operate in climacteric plants (Barry et al., 2000). System 1 functions during normal vegetative growth, is ethylene autoinhibitory, and is responsible for producing basal ethylene levels that are detected in all tissues, including those of nonclimacteric fruits. System 2 operates during the ripening of climacteric fruit. It has been proposed that tomato ACS1 and ACS6 are involved in the production of system 1 ethylene in green fruits (Barry et al., 2000). System 1 continues until the fruit ripening initiates, when *ACS1* expression increases and *ACS2*, *ACS4*, *ACO2*, and *ACO4* are induced as a result of positive feedback regulation (Nakatsuka et al., 1998; Barry et al., 2000). We found that *ACS6*, but not the other ACS genes, together with *ACO4* and two of the ethylene receptor genes, *ETR5* and *ETR6*, as well as the transcription factor gene *EIL2* are induced in the *AP2i* fruits, suggesting that *AP2a* is a negative regulator of their expression. A model was proposed for tomato fruit in which preclimacteric system 1 ethylene is mediated via constitutively expressed *ACS1* and *ACS3* and negatively regulated through a feedback loop involving *ACS6* (Nakatsuka et al., 1998). Our data suggest that AP2a might be a regulator of the switch between the two ethylene systems (1 and 2) through negative regulation of *ACS6* expression. The other genes upregulated in *AP2*i fruits, the *ETR6* receptor gene and *ACO4* (Nakatsuka et al., 1998; Kevany et al., 2007), were shown to be regulated by elevated ethylene levels, which could be the reason for their higher expression in the *AP2*i fruits, as opposed to regulation by AP2a directly. Since the expression of both *ETR5* and *EIL2* is not ethylene dependant (Tieman et al., 2001; Kevany et al., 2007), their expression could be under direct regulation of AP2a. Finally, we also found tomato *EBF1*, a homolog of *Arabidopsis EBF1*, to be upregulated in *AP2i* fruits. EBF1 is involved in targeting EIN3 for degradation and thus modulates the ethylene response (Guo and Ecker, 2003). The two tomato homologs, *EBF1* and *EBF2*, are regulated by ethylene and auxin, and their silencing causes a constitutive ethylene response, plant senescence, and early fruit ripening (Yang et al., 2010).

In addition to ripening, ethylene is also known to be involved in other processes, such as pathogen and wounding responses, leaf senescence, and the abiotic and biotic stress response (for review, see Alexander and Grierson, 2002). In *AP2*i fruits, a large group of genes encoding pathogenesis-related proteins (PR proteins and chitinases), as well as one key regulator of the biotic stress response, the MYB transcription factor gene *AIM1* (Abuqamar et al., 2009), are upregulated. Many of these genes are also likely upregulated in *Cnr* mutant fruits (Eriksson et al., 2004). Our data suggest that in wild-type fruits, *AP2a* acts not only as repressor of ethylene biosynthesis, but also as a repressor of ethylene-induced pathogen defense and wounding responses.

In comparison with ethylene, very little is known about the role of other hormones in fruit ripening. The role of auxins has been extensively investigated in other fruits, such as strawberry (*Fragaria* × *ananassa*) (Harpster et al., 1998) and grape (*Vitis vinifera*) (Davies et al., 1997). Our transcriptomics data show a high level of repression of *GH3*, which is involved in maintaining auxin homeostasis by conjugating excess indole-3-acetic acid to amino acids (Staswick et al., 2005). In addition to *GH3*, *ARF4*, a transcription factor gene involved in auxin signaling, was suppressed. *ARF4* (also known as *DEVELOPMENTALLY REGULATED12* [*DR12*]; Jones et al., 2002) is developmentally regulated, and its expression increases in fruits during ripening and decreases after ethylene treatment of immature fruits. Knockdown of *DR12* in transgenic tomato plants caused blotchy fruit ripening, similar to the defects observed here for *AP2* RNAi plants, suggesting that at least part of the effects of *AP2*a

downregulation is caused by deregulation of *ARF4*. Genes involved in the biosynthesis of brassinosteroid hormones, like *DWARF* (Nomura et al., 2005) and the homolog of *Arabidopsis DWF1*/*DIMINUTO* (Klahre et al., 1998), were downregulated in the *AP2*i fruits. Previously, it was shown by treatment of tomato pericarp discs with brassinosteroids that these could accelerate ripening and increase ethylene production (Vidya Vardhini and Rao, 2002). Although brassinosteroids recently have been shown to play a role in early fruit development in cucumber (*Cucumis sativus*) (Fu et al., 2008) and in fruit ripening in grape (Symons et al., 2006), their function in tomato fruit development or ripening remains to be determined.

Effect of AP2a Downregulation on Fruit Metabolism

From the gene ontology analysis of our transcriptomics data, it is apparent that most of the metabolic pathways that are significantly regulated are related to primary metabolism. In agreement with these data are the changes in the levels of accumulation of different primary metabolites that were measured in the *AP2*i fruits compared with the wild-type ripe fruits. We found that *AP2a* positively regulates the accumulation of Leu and Ile, probably through regulation of *BCAT1*. *BCAT1* transcripts were shown to accumulate at the highest level in ripening fruits, and BCAT1 is likely the primary enzyme involved in the recycling of branched-chain amino acids generated by protein degradation. This statement is in agreement with data obtained in a recent study of the tomato *BCAT* gene family (Maloney et al., 2010).

Taken together, our metabolic and transcriptomics data indicate that the downregulation of AP2a transcription factor has a major effect on cell wall metabolism, which is associated with fruit softening and shelf life and the biosynthesis of secondary metabolites and their primary metabolite precursors, as well as decreased flux through the tricarboxylic acid cycle.

Additionally, the downregulation of *LOXC* may well affect volatile production in *AP2* RNAi plants during ripening. The chromoplast-located TomloxC protein has been shown to be involved in the production of flavor volatiles hexanal, hexenal, and hexenol from linoleic and linolenic acid, which may be produced through thylakoid membrane disruption at the transition from chloroplasts to chromoplasts (Chen et al., 2004).

AP2a Participates in a Transcriptional Regulatory Network That Regulates Tomato Fruit Ripening

Unlike *Arabidopsis AP2*, which is mostly expressed in flowers and functions in floral organ identity, tomato *AP2a* has acquired a novel function in fleshy fruit development. Some of the other tomato *AP2* paralogs may still be involved in floral organ identity as A-class homeotic genes, like AP2 in *Arabidopsis* (Bowman et al., 1991; Coen and Meyerowitz, 1991); however, further research is needed to unravel the function of these other tomato genes.

The AP2a transcription factor appeared to have dual functions in tomato fruit ripening as a positive and negative regulator of different processes occurring during ripening. An interesting observation was that AP2a most probably functions downstream of known major positive regulators of fruit ripening, such as NOR, RIN, and CNR. At least in the case of CNR, this regulation is likely to be direct, since CNR is able to bind directly to a promoter element of *AP2a*. On the other hand, in the fruit pericarp, *AP2a* regulates the expression of *CNR* in a negative manner. In both the *Cnr* (Eriksson et al., 2004) mutant and in *AP2*i transgenic fruits, several ripening-associated genes are downregulated, such as the carotene synthesis pathway genes, *LOXB* and *LOXC*, pectin methylesterase, and *EXP3*, indicating that *AP2a* has positive ripening regulatory functions besides its negative regulatory function in ethylene synthesis. Further research, such as the identification of direct and indirect targets of the key transcription factors, will likely contribute to further mapping of the transcriptional network that regulates fruit ripening, a process in which AP2a plays an essential function.

METHODS

Greenhouse/Growth Conditions

Transgenic plants were transferred to soil and grown in the greenhouse at ambient temperatures (>20°C) under natural light supplemented with artificial sodium lights, according to a 16-h-light/8-h-dark cycle. Standard greenhouse culture conditions, with regular fertilizer application, were used.

Construction of RNAi Construct and Transgenic Tomato Plants

The sequences, and associated names, available on the Tomato Functional Genomics Database and The Institute for Genomic Research (TIGR) websites, are always being updated; however, old names remain linked to updated information. Therefore, the original names first notated for this research are kept for the article. The primers used (see Supplemental Table 3 online) for the RNAi construct were designed based on the sequence of EST311591 (GenBank AW442195; http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=tomato). RT-PCR was performed with red ripe fruit RNA, using an annealing temperature of 54°C, and the products were cloned into pENTR/D-TOPO (Invitrogen) (resulting plasmid designated pARC131 and CZN098 for specific RNAi fragment) and subcloned into pHellsgate8 (designated pARC139 and CZN096, respectively) (Helliwell et al., 2002). pARC139 and CZN096 were transformed into electrocompetent *Agrobacterium tumefaciens* strain EHA105, followed by transformation into *Solanum lycopersicum* cv Moneymaker and cv M82, respectively, as described by van Roekel et al. (1993). Tissue culture-generated nontransgenic Moneymaker and M82 plants were used for wild-type controls. Sixteen independent transgenic *AP2*i-1 and 14 independent transgenic *AP2*i-2 RNAi lines were generated and analyzed for ploidy level by flow cytometry as described elsewhere (de Laat et al., 1987), and polyploid plants were removed from the population.

Plasmid Constructions

AP2a and *CNR* were amplified from the cDNA made from mixed fruit stages using the primer pairs described in Supplemental Table 4 online. The full-length open reading frames of *AP2a* and *CNR* were inserted into the pENTR-D TOPO vector following the Gateway protocol (Invitrogen). All plasmids were controlled by sequence analyses (DETT sequence kit; Amersham). Finally, expression vectors for the colocalization analysis were obtained by LR reaction following the Gateway protocol. For the C-terminal yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) fusions, CZN576 and CZN575 destination vectors, respectively, were used. For constructing CZN575, the open reading frame from sCFP3A was amplified from pSCFP3A-C1 (Kremers et al., 2006) and was

cloned into pGD120 (Immink et al., 2002). The obtained product was digested with *Xba*I, which is located just before the start codon of sCFP3A, and the overhangs were blunted with Klenow. Subsequently, a Gateway conversion cassette (Invitrogen) was ligated into this vector, resulting in the destination vector CZN575, containing the expression cassette pCaMV35S:Gateway-sCFP3A:NOS terminator. For the CZN576 vector, the open reading frame from sYFP2 was amplified from pSYFP2-C1 (Kremers et al., 2006), and the remaining cloning strategy was identical to that used for the CZN575 vector.

For the N-terminal GST fusion, the full-length CNR PCR fragment was obtained with the primers listed in Supplemental Table 3 online. The purified PCR fragment was digested with *Eco*RI and *Not*I and ligated into pGEX4T1. The resulting construct was verified by sequencing and transformed into *Escherichia coli* Arctic Express strain (Stratagene). The protein expression and GST purification were performed as described before (Karlova et al., 2009).

For the electrophoretic mobility shift assay or gel retardation assay, the two *AP2a* promoter regions chosen were PCR amplified with the primers listed in Supplemental Table 3 online, purified, and ligated into the pGEMT vector (Promega).

Ethephon Treatment of Tomato Fruit

Ethephon (Acros Organics) treatment of intact mature green fruits involved a 1-min dip in 50 mM fresh aqueous ethephon solution for 6 h.

Gel Retardation Assays

The binding reactions for the gel retardation assays were performed as described by Winter et al. (2002). Fifty nanograms of in vitro GST-CNR purified protein were used per binding reaction. PCR fragments from the above described promoter fragment construct were biotinylated, and 40 nM of probe was used per lane. After native polyacrylamide gel electrophoresis, the DNA was blotted to positively charged nylon membranes (Hybond N^+; Amersham), and signal was detected using the chemiluminescent nucleic acid detection module (Peirce).

Images

Micrographs of seeds were taken on a Zeiss STEMI SV8 microscope. Seed hairs were processed and viewed using a JEOL JSM-6330F field emission electron scanning microscope as described elsewhere (Angenent et al., 1995).

Fluorescence Microscopy in Living Cells

Arabidopsis thaliana leaf protoplasts were transfected as described by Aker et al. (2006). Plasmid DNA (15 μg) was used and the protoplasts were incubated overnight at 25°C before imaging. Images were acquired using an upright confocal laser scanning microscope (Leica SPE DM5500) with a 10 × 0.30 CS ACS APO (air) lens, using the LAS AF 1.8.2 software (Leica).

Quantitative Real-Time PCR

RNA isolation from fruits or pericarp of the fruits was performed with the InviTrap Spin Plant RNA kit (Invitek) according to the manufacturer's protocol and from all other tissues by the Trizol method (Sigma-Aldrich), except from seeds, where a modified method was used. Seeds were ground in a 5:5:1 mixture of buffer (180 mM Tris pH 8.2, 90 mM LiCl, 4.5 mM EDTA, 1% SDS, and 0.1% β-mercaptoethanol), phenol, and chloroform. After phase separation by centrifugation, the watery phase was reextracted with 1 mL phenol/chloroform (1:1) and subsequently with chloroform. The extracted RNA was precipitated with one-third volume of 8 M LiCl overnight at −20°C. Precipitated RNA was centrifuged, washed with 70% ethanol, dried, and dissolved in RNase-free water.

DNaseI-treated RNA was reverse transcribed (Applied Biosystems), and 1 μL of cDNA solution was used for the real-time PCR analysis. Real-time qPCR was performed with iQ SYBR Green Supermix (Bio-Rad) using the iCycler iQ5 system (Bio-Rad) and gene-specific primers (see Supplemental Table 4 online). Two independent biological replicates were analyzed per sample (or two to three fruits from the same plant were mixed together per sample). Relative quantification of specific mRNA levels was performed using the cycle threshold (Ct) $2^{-\Delta(\Delta Ct)}$ method (Software IQ5 2.0; Livak and Schmittgen, 2001). Expression values were normalized using the housekeeping gene actin or CAC and TIP41 in case of sepals, petals, and seeds (Expósito-Rodriguez et al., 2008) from tomato.

Ethylene Measurements

Fruits at breaker or breaker +7 d stages were harvested and placed in open 500-mL jars for 2 h. Jars were then sealed and incubated at room temperature for 35 min, and 1.5 mL of headspace gas was injected into a Focus GS gas chromatograph (Thermo-Electron) equipped with a flame ionization detector. Samples were compared with reagent grade ethylene standards of known concentration and normalized for fruit weight.

Carotenoid Analysis

Pooled fruits from independent plants were harvested and homogenized. Aliquots from these samples were taken separately for extraction and analysis of primary and secondary metabolite content by HPLC coupled with photodiode array detection and MS. The extraction, HPLC separation, photodiode array detection, and quantification of carotenes, carotenoids, and xanthophylls were previously described (Fraser et al., 2000). In this study, 0.5 to 1 g of frozen fruit tissue was extracted with chloroform and methanol (2.5:1 by volume), mixed, and incubated for 20 min on ice. Then, 1 volume of deionized water was added. A partition was formed after mixing by centrifugation. The organic hypophase was removed, and the aqueous phase was reextracted with chloroform (2.5 times by volume). HPLC separations were performed using a C30 reverse-phase column (250 × 4.6 mm) purchased from YMC. The mobile phases used were methanol (A), water/methanol (20/80 by volume) containing 0.2% ammonium acetate (B), and tert-methyl butyl ether (C). The gradient used was 95% A:5% B, isocratically for 12 min and then stepped to 80% A:5% B:15%C, from which a linear gradient to 30% A:5% B:65% C over 30 min was performed. A Waters Alliance model 2695 injection and solvent delivery system was used. Detection was performed continuously from 220 to 700 nm with an online photodiode array detector (Waters 966). Identification was performed by cochromatography and comparison of spectral properties with authentic standards and reference spectra. Annotated chromatograms of this HPLC system are provided elsewhere (Fraser et al., 2007). Quantitative determination of carotenoids was performed by comparison with dose–response curves (0.2 to 1.0 mg) constructed from authentic standards. Purchasing, preparation, and characterization of authentic standards are as described previously (Fraser et al., 2000).

GC-MS Analysis

Metabolite extraction, derivatization, GC-MS analysis, and data processing were performed as described previously (Roessner et al., 2001; Lisec et al., 2006). Metabolites were identified in comparison to database entries of authentic standards (Kopka et al., 2005; Schauer et al., 2005).

Peptide Antibody Generation

For polyclonal AP2a antibodies, the peptide CRTQNNGFHHYFMRP was generated and used for rabbit immunization by Genosphere Biotechnologies.

Protein Gel Blot Analysis

Plant material (tomato fruits) was ground in liquid nitrogen, and 0.2 g was dissolved in sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% bromphenol blue, 20% glycerol, and 100 mM DTT) and boiled for 5 min. Protein gel blotting was performed as described before (Karlova et al., 2006). Proteins were detected with anti-AP2 antibodies (2000× diluted).

Phylogenetic Analysis

Protein homology searches in the National Center for Biotechnology Information (NCBI) GenBank protein database were done using the BLASTP algorithm (Altschul et al., 1997). Homology searches in the TIGR Tomato Gene Index were done with the TBLASTN algorithm. Multiple protein alignments of the full-length proteins were produced with the ClustalW program of the DNAstar Megalign software package (DNASTAR). Phylogenetic trees were produced with the ClustalX package (Thompson et al., 1997). Phylogenetic reconstruction was obtained by the neighbor-joining method (Saitou and Nei, 1987) together with bootstrap analysis using 1000 replicates. Kimura correction for multiple substitutions was applied (Kimura and Takahata, 1983). The tree was visualized using the TreeView package (Page, 1996).

Microarray Analysis

Transcript profiling was performed on fruits of wild-type tomato and *AP2*-1 RNAi mutant lines, at the breaker +7 d stage (U.S. standards for grades of fresh tomatoes, 1991). RNA was isolated with the Trizol method (Sigma-Aldrich) and hybridized to the Syngenta Tomato Affymetrix GeneChip. Three biological replicates and two technical replicates were analyzed from either unmodified tomato variety Moneymaker or from an *AP2*-1 RNAi line. Twelve experiments (slides) were analyzed. Data were analyzed using the GeneData Expressionist suite of tools. All 12 experiments passed quality controls and were used for analysis. The Bioconductor RMA algorithm was used for background subtraction according to a Bayesian model using the PM Features Only option ('bgversion = 2' in the BioConductor routine) for quantile normalization of the arrays (Bolstad et al., 2003) and for condensing probes into probe regions (hereafter referred to as genes) (Irizarry et al., 2003).

A Welch *t* test was performed to identify genes with significant differences in expression level between the two data sets. P values were adjusted for false discovery rate 0.05, as described by Benjamini and Hochberg (1995).

Accession Numbers

Sequence data from this article can be found in Swiss-Prot or Protein Bank/NCBI databases under the following accession numbers: *Arabidopsis* ANT protein, Q38914; At RAP27/TOE1, Q9SK03; At TOE2, Q9LVG2; At SMZ, Q6PV68; At SNZ, Q6PV67; At TOE3, Q9FH95; At AP2, AAC13770; At WRI1, NP_191000; At AIL1, Q1PFE1; At AIL5, Q6PQQ3; At AIL6, Q52QU2; At AIL7, Q6J9N8; At BBM, Q6PQQ4; At PUCHI, NP_197357; At PLT1, Q5YGP8; At PLT2, Q5YGP7; Tomato AP2a, ACD62792, Sl AP2c, HQ586951, Sl AP2b, HQ586952, Sl AP2d, HQ586953; Sl AP2e, HQ586954; *Petunia* PHAP2A, AAD39439, and PHAP2B, AAD39440; *Antirrhinum* LIP1, AAO52746 and LIP2, AAO52747; *Malus domestica* AHAP2, AAL57045; *Zea mays* GLOSSY15, AAC49567; IDS1, NP_001104904. The accession numbers of genes used in the microarray analysis are provided in Table 1 and Supplemental Data Set 3 online.

Supplemental Data

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

Supplemental Figure 1. Phylogenetic Tree of the AP2 Subfamily, with the AP2 and ANT Subgroups Indicated.

Supplemental Figure 2. Protein Sequence Alignment and Open Reading Frame Nucleotide Sequence of AP2a and Its Homologs.

Supplemental Figure 3. Decreased Expression of AP2a Protein Results in Alterations in Sepal Morphology.

Supplemental Figure 4. Decreased Expression of AP2a Protein Results in Alterations in Fruit and Seed Morphology.

Supplemental Figure 5. Schematic Representation of the Metabolic Changes Occurring in the *AP2i*-1 Silenced Ripe Fruits Compared with the Wild Type.

Supplemental Figure 6. Relative Expression of *RIN* and *TAGL1* in Wild-Type and *AP2*i-1 Breaker Fruits.

Supplemental Table 1. Pathways Significantly Altered in *AP2*i Fruits.

Supplemental Table 2. Metabolite Levels in Wild-Type and *AP2*i Transgenic Fruits.

Supplemental Table 3. Oligonucleotides Used in This Study.

Supplemental Data Set 1. FASTA Format File of the Alignment Used for Creating Figure 1A.

Supplemental Data Set 2. FASTA Format File of the Alignment Used for Creating Supplemental Figure 1.

Supplemental Data Set 3. Gene Expression Changes in *AP2* RNAi Ripe Fruits.

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