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LARGE-SCALE BIOLOGY ARTICLE

Transcriptional Regulation of Fruit Ripening by Tomato FRUITFULL Homologs and Associated MADS Box Proteins[W]

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The tomato (*Solanum lycopersicum*) MADS box FRUITFULL homologs FUL1 and FUL2 act as key ripening regulators and interact with the master regulator MADS box protein RIPENING INHIBITOR (RIN). Here, we report the large-scale identification of direct targets of FUL1 and FUL2 by transcriptome analysis of *FUL1*/*FUL2* suppressed fruits and chromatin immunoprecipitation coupled with microarray analysis (ChIP-chip) targeting tomato gene promoters. The ChIP-chip and transcriptome analysis identified FUL1/FUL2 target genes that contain at least one genomic region bound by FUL1 or FUL2 (regions that occur mainly in their promoters) and exhibit *FUL1*/*FUL2*-dependent expression during ripening. These analyses identified 860 direct FUL1 targets and 878 direct FUL2 targets; this set of genes includes both direct targets of RIN and nontargets of RIN. Functional classification of the FUL1/FUL2 targets revealed that these FUL homologs function in many biological processes via the regulation of ripening-related gene expression, both in cooperation with and independent of RIN. Our in vitro assay showed that the FUL homologs, RIN, and tomato AGAMOUS-LIKE1 form DNA binding complexes, suggesting that tetramer complexes of these MADS box proteins are mainly responsible for the regulation of ripening.

INTRODUCTION

During ripening, fruits undergo biochemical and physiological changes in flesh texture, pigmentation, sugar contents, aroma, and nutritional qualities. Understanding the mechanisms that regulate fruit ripening can help efforts to improve fruit quality. The genetically programmed system that regulates ripening of fleshy fruits includes phytohormone signaling pathways and transcription factor networks, acting both cooperatively and independently (Giovannoni, 2007; Gapper et al., 2013). For example, ethylene promotes ripening in climacteric fruits such as tomato (*Solanum lycopersicum*), banana (*Musa* spp), and apple (*Malus domestica*).

In the transcription factor networks that affect fruit ripening, MADS box family transcription factors serve as central regulators of ripening (Smaczniak et al., 2012a; Gapper et al., 2013). MADS box transcription factors, which contain a conserved DNA binding domain (MADS domain), are widely present in eukaryotes. In plants, MADS box proteins (type II MIKC^C-type) play an essential role in almost every developmental process, including fruit development and ripening (Smaczniak et al., 2012a). The tomato MADS box gene *RIPENING INHIBITOR* (*RIN*), a homolog of *Arabidopsis thaliana SEPALLATA* (*SEP*), is one of the earliest acting key factors that regulate ripening, including both ethylene-dependent and -independent processes (Vrebalov et al., 2002). The *rin* mutants exhibit a severe ripening-defective phenotype in which the fruits fail to soften, do not accumulate lycopene, and do not show a climacteric rise of respiration and ethylene production (Tigchelaar et al., 1978). Other climacteric fruit species, and even the nonclimacteric fruit strawberry (*Fragaria* × *ananassa*), have *SEP* homologs involved in fruit ripening (Elitzur et al., 2010; Seymour et al., 2011; Ireland et al., 2013; Schaffer et al., 2013).

In addition to *RIN*, the tomato MADS box genes *AGAMOUS-LIKE1* (*TAGL1*), *FRUITFULL1* (*FUL1*; formerly named *TDR4*), and *FUL2* (formerly *MBP7*) act as fruit ripening regulators (Itkin et al., 2009; Vrebalov et al., 2009; Giménez et al., 2010; Pan et al., 2010; Bemer et al., 2012; Shima et al., 2014). *TAGL1* and *FUL1*/*FUL2* are homologs of *Arabidopsis SHATTERPROOF* (*SHP*) and *FUL*, respectively, and their suppression results in ripening-defective phenotypes partly similar to the phenotype of *rin* mutant fruits. Also, a bilberry (*Vaccinium myrtillus*) *FUL* homolog, Vm-*TDR4* (Jaakola et al., 2010), and a strawberry *SHP* homolog, Fa-*SHP* (Daminato et al., 2013), are associated with fruit ripening. The identification of ripening-related MADS box proteins opens opportunities for the study of ripening regulation. However, it remains less clear how these MADS box factors coordinately regulate fruit ripening, compared with regulation of flower or dry-type fruit (e.g., *Arabidopsis*) development, where *SEP*, *SHP*, and *FUL* have well-characterized genetic and molecular functions (Gu et al., 1998; Liljegren et al., 2000; Pelaz et al., 2000; Honma and Goto, 2001; Kaufmann et al., 2009; Robles et al., 2012; Smaczniak et al., 2012b).

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To elucidate the transcriptional regulation mechanism of fruit ripening, several studies have identified direct targets of the master regulator RIN; these targets include the ripening-regulating genes *FUL1*, *NON-RIPENING* (*NOR*; NAC family transcription factor), and *COLORLESS NON-RIPENING* (*CNR*; SQUAMOSA promoter binding protein-like family transcription factor) (Fujisawa et al., 2011, 2012; Martel et al., 2011; Qin et al., 2012). Large-scale analyses identified additional direct RIN target genes (Fujisawa et al., 2013; Zhong et al., 2013), revealing that RIN directly regulates a broad range of biological processes and metabolic pathways, including ripening-related ethylene production and lycopene accumulation during ripening. Also, the tomato FUL homologs, which interact with RIN to form DNA binding protein complexes, play a crucial role in fruit ripening (Leseberg et al., 2008; Martel et al., 2011; Bemer et al., 2012; Shima et al., 2013). The FUL homologs act upstream of the ethylene signaling pathway during ripening, as shown by a phenotypic analysis of *FUL1*/*FUL2*-suppressed tomato fruits (Shima et al., 2014).

In this study, to understand the biological roles of the FUL homologs, we identified direct FUL1 and FUL2 target genes by transcriptome analysis of *FUL1*/*FUL2*-suppressed fruits and chromatin immunoprecipitation coupled with microarray analysis (ChIP-chip). The combined ChIP-chip and transcriptome analyses successfully identified direct target genes of FUL1 and FUL2 on a large scale. The comparison of FUL1, FUL2, and RIN target genes suggests that the FUL homologs regulate transcription in both RIN-associated and RIN-independent manners. We also used an in vitro assay to examine the interactions of RIN, FUL1, FUL2, and TAGL1. Our results suggest that these ripening-related MADS box proteins form higher order complexes that are responsible for the main part of ripening regulation.

RESULTS

Transcriptome Profiling of *FUL1*/*FUL2*-Suppressed Fruits by RNA Sequencing

To identify downstream targets of FUL1 and FUL2, we first examined the alterations in transcription caused by knockdown of *FUL1* and *FUL2*. We previously used RNA interference (RNAi) in transgenic tomato plants to cosuppress *FUL1* and *FUL2* (Shima et al., 2014). In this study, we used fruits of a strong *FUL1*/*FUL2*-suppressed transgenic line, TF18, which exhibits a ripening-defective phenotype with reduced ethylene production and lycopene accumulation (Shima et al., 2014), for transcriptome analysis.

To obtain a transcriptome profile of *FUL1*/*FUL2*-suppressed fruits, we conducted massively parallel RNA sequencing (RNA-Seq) analysis on TF18 fruits harvested at 35 d after pollination (DAP), the same age as the preripening (mature green) stage of the wild-type fruits, which we term "G age." We also examined fruits at 45 DAP, the same age as the ripening (pink coloring) stage of the wild-type fruits, which we term "P age." For transcriptome comparison, we also analyzed fruits of wild type (Ailsa Craig cultivar [AC]) and a *rin* mutant. We validated the RNA-Seq data (Supplemental Table 1 and Supplemental Data Set 1) by quantitative RT-PCR (qRT-PCR) for nine known ripening-related genes, which showed good correlation between the two assays (Supplemental Figure 1). The RNA-Seq data showed that the upregulation of many well-characterized ripening-induced genes during wild-type ripening (represented by the lane AC_P/AC_G in Figure 1A) was suppressed in the P age TF18 fruits (TF18_P/AC_P in Figure 1A). The suppression was stronger than in P age *rin* mutant fruits (*rin*_P/AC_P in Figure 1A). Multidimensional scaling (MDS) analysis of the RNA-Seq data showed a similar tendency, placing the TF18 samples (at G and P ages) on the map far away from the AC and *rin* samples, which were placed near each other (Figure 1B). These results indicate that the *FUL1*/*FUL2* suppression affects gene expression (both at the G and P ages) more strongly than the *rin* mutation.

Detection and Functional Classification of FUL1/FUL2-Regulated Genes

To identify FUL1/FUL2-regulated genes and clarify the role of the FUL homologs during ripening, we identified differentially expressed genes (DEGs) between samples using the RNA-Seq data. To do this, we applied the cutoff value of fold change (FC) ratio >2 or <0.5 with false discovery rate (FDR) ≤0.05 for genes that are substantially expressed in either or both samples (average counts per million reads ≥ 5). Comparison of the P age AC and TF18 fruits identified 5953 DEGs, including 2856 upregulated and 3097 downregulated by *FUL1*/*FUL2* suppression (DEGs for *FUL*; Supplemental Data Set 2A). Similarly, comparison of the P and G age AC fruits identified 5978 DEGs, including 3003 upregulated and 2975 downregulated with ripening (DEGs for ripening; Supplemental Data Set 2B). Comparison of the P age AC and *rin* fruits identified 3821 DEGs, including 2399 upregulated and 1422 downregulated by the *rin* mutation (DEGs for *rin*; Supplemental Data Set 2C). The set of DEGs for *FUL* included 4809 (80%) of the DEGs for ripening, but the set of DEGs for *rin* included markedly fewer DEGs for ripening (1927 genes; 32%) (Supplemental Figure 2).

To provide an overview of the roles of the FUL homologs, we classified the DEGs for *FUL* based on the functions of their *Arabidopsis* homologs, as predicted by the Munich Information Center for Protein Sequences (MIPS) functional catalog database (Supplemental Data Set 2A). We found categories and subcategories (e.g., protein synthesis and differentiation of organ, tissue, and cell type) in which the frequency of genes in the up- and downregulated subsets of DEGs for *FUL* is significantly overrepresented ($P < 0.001$ by Fisher's exact test) compared with the whole genome (Supplemental Data Set 3). Both the up- and downregulated subsets of DEGs for *FUL* had more overrepresented categories than the up- and downregulated subsets of DEGs for *rin*, respectively (Supplemental Data Set 3). Typical ripening-associated categories such as ethylene production (metabolism of Met) and carotenoid metabolism (isoprenoid metabolism and tetraterpene metabolism) were overrepresented in the downregulated subset of DEGs for *rin* but not in that of the DEGs for *FUL*, although many genes in these categories were included in the subset of DEGs for *FUL* (Supplemental Data Sets 2C and 3). The results suggest that the FUL homologs regulate expression of more diverse genes than RIN, which mainly regulates genes involved in ripening.

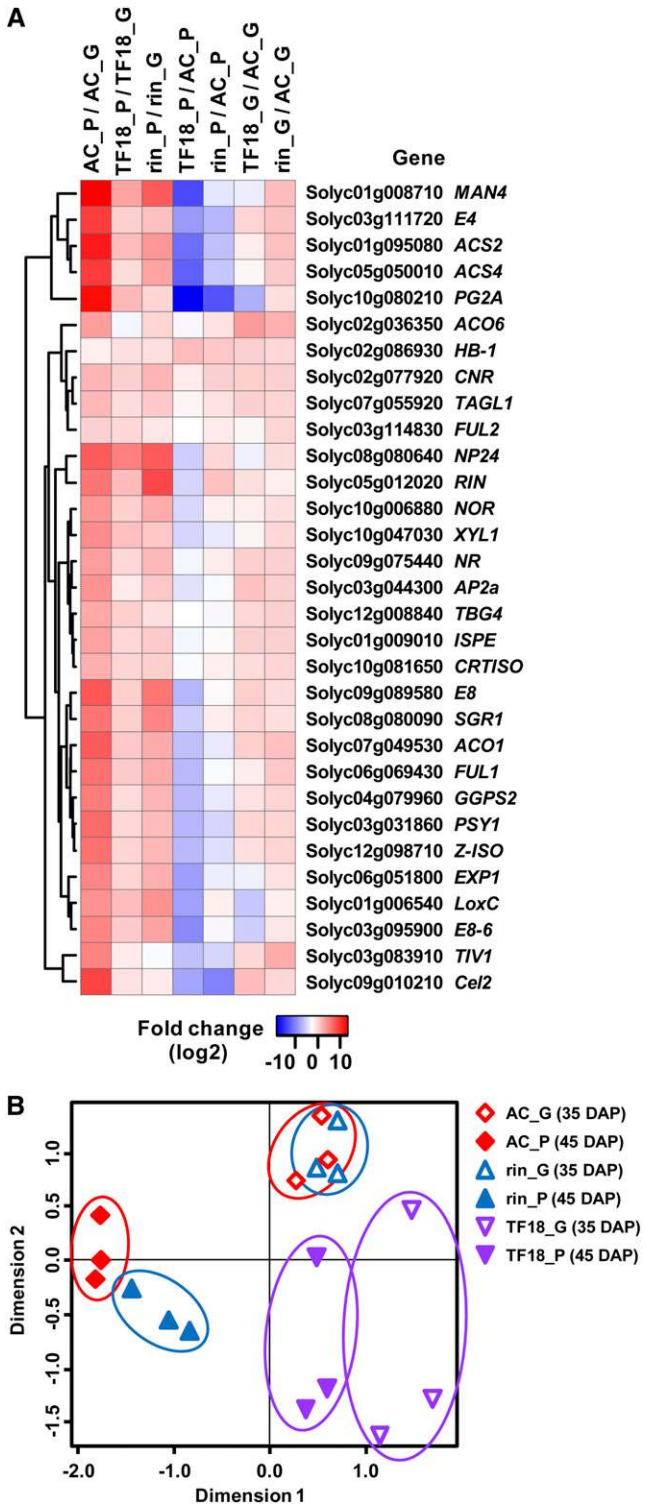

Figure 1. Transcriptome Profiling of the *FUL1*/*FUL2*-Suppressed Tomato Fruits.

(A) Expression of representative ripening-related genes in the wild type (AC), the *rin* mutant, and the *FUL1*/*FUL2*-suppressed line (TF18) fruits. The samples harvested at 35 and 45 DAP are represented by G and P, respectively (see text). Sample pairs compared are shown above the heat map. *ACO1* and *ACO6*, genes for ACC oxidase; *ACS2* and *ACS4*, genes for ACC synthase; *AP2a*, an *APETALA2* homolog gene; *Cel2*, a gene for endo-β-1,4-glucanase; *CRTISO*, a gene for carotenoid isomerase; *E4*, a gene for Met sulfoxide reductase; *E8* and *E8-6*, genes for ACC oxidase homologs; *EXP1*, a gene for expansin; *GGPS2*, a gene for geranylgeranyl diphosphate synthase; *HB-1*, a gene for HD-zip homeobox protein; *ISPE*, a gene for 4-diphosphocytidyl-2-*C*-methyl-D-erythritol kinase; *LoxC*, a gene for lipoxygenase; *MAN4*, a gene for mannan endo-1,4-β-mannosidase; *NP24*, a gene for osmotin-like protein; *NR*, *NEVER RIPE*; *PSY1*, a gene for phytoene synthase; *PG2A*, a gene for polygalacturonase; *SGR1*, a gene for stay-green protein; *TBG4*, a gene for β-galactosidase; *TIV1*, a gene for acid β-fructofuranosidase (invertase); *XYL1*, a gene for β-D-xylosidase; *Z-ISO*, a gene for ζ-carotene isomerase.

(B) MDS analysis of RNA-Seq data from the fruits of AC, *rin* mutants, and the *FUL1*/*FUL2*-suppressed line (TF18). Each spot represents an individual sample, with three biological replicates, indicated by the name of each line, and followed by the age harvested (G or P).

Detection of FUL1- and FUL2-Bound Genomic Regions by ChIP-chip

To detect the genomic regions bound by FUL1 and FUL2 in tomato promoters during ripening, we performed a ChIP-chip analysis. We used ChIP to collect DNA fragments bound to FUL1 and FUL2 from ripening tomato fruits at the pink coloring stage using antibodies to FUL1 and FUL2. The ChIPed and input (no ChIP) DNAs were hybridized to the NimbleGen microarrays, which contain probes designed from 2-kb promoter regions of all predicted tomato genes (ITAG2) and were previously used to identify direct RIN targets (Fujisawa et al., 2013). Hybridization signals were analyzed to assign each probe a $\log_2$-scale FC value of the ChIPed DNA relative to the input DNA. Consecutive probes with high average FC from three biological replicates at statistically significant levels (FDR $\leq$ 0.05; for more details, see Fujisawa et al., 2013) defined ChIP-chip peak regions (Supplemental Table 2). This analysis identified 2307 FUL1-bound regions and 2457 FUL2-bound regions (Figure 2; Supplemental Data Sets 4A and 4B). We validated the microarray data by quantitative ChIP-PCR (qChIP-PCR) for the mapped regions with the 10 highest scores each for FUL1 and FUL2. The qChIP-PCR confirmed >4-fold enrichment of all the regions analyzed, although we found no significant correlation between the ChIP-chip scores and the qChIP-PCR enrichment levels (Supplemental Figure 3), possibly due to the difference in dynamic range between the two assays.

Overlap and Motif Conservation of DNA Binding Regions of FUL1, FUL2, and RIN

We previously reported that in several ripening-related gene promoters, the binding regions of FUL1 and FUL2 overlap with each other and with RIN-bound regions (Shima et al., 2013). To examine this in more loci, we analyzed the overlaps of FUL1- and FUL2-bound regions and RIN-bound regions (1217 regions) that we identified by reanalysis of our previously examined ChIP-chip data (Fujisawa et al., 2013) with the same algorithm we used to identify the genomic regions bound by the FUL homologs. We defined an overlapping region as one factor's bound region that, at least in part, is detected as the binding region of another

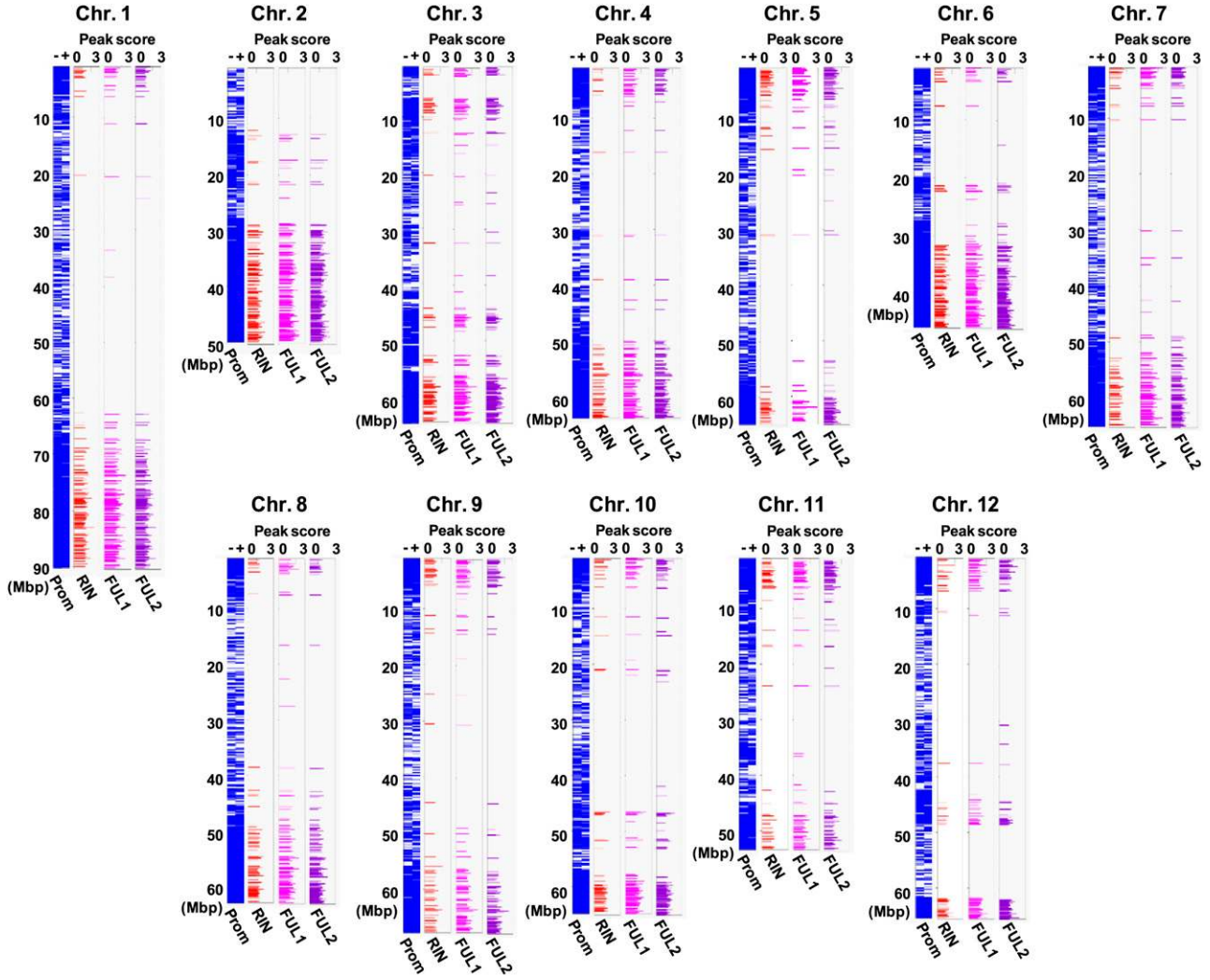

Figure 2. Distribution of FUL1- and FUL2-Bound Regions on the Tomato Chromosomes.

Genomic positions of binding regions of FUL1 and FUL2 detected by ChIP-chip on the 12 chromosomes are indicated by bars in magenta and violet, respectively, with the $\log_2$ scale peak score. For comparison, the positions of RIN-bound regions identified in this study are indicated in red. Positions of the promoters (+, forward strand; −, complementary strand) where ChIP-chip probes were designed are indicated by blue bars.

factor(s). As summarized in Supplemental Table 3, 1145 (50%) of FUL1-bound and 1134 (46%) of FUL2-bound regions overlapped, at least in part, with those of RIN. Furthermore, 1810 (78%) of FUL1-bound regions and 1803 (73%) of FUL2-bound regions overlapped with each other. By contrast, 435 (19%) of FUL1-bound, 604 (25%) of FUL2-bound, and 39 (3.2%) of RIN-bound regions did not overlap with the other transcription factor bound regions. To verify that FUL1, FUL2, and RIN independently bind these nonoverlapping regions, we compared these regions with the regions detected by each of the three replicate ChIP-chip assays for the other factors. The results showed that 58 to 68% for FUL1, 55 to 70% for FUL2, and 36% for RIN of their nonoverlapping regions were not found in the other factors' bound regions in any replications, confirming that at least these regions are most likely unique to each factor (Supplemental Table 4). By contrast, considering the variation in ChIP-chip between replicates (Supplemental Table 2), the remaining regions might include undetected common regions (Supplemental Table 4).

To clarify DNA binding specificities of FUL1, FUL2, and RIN, we compared the nonoverlapping regions of each transcription factor based on the frequency of four types of CArG motif sequences: PAL type [$CC(A/T)_6GG$], N10 type [$CTA(A/T)_4TAG$] (West et al., 1998), and typical RIN binding motifs [$C(C/T)(A/T)_6(A/G)G$ (Ito et al., 2008) and $C(A/T)_8G$ (Fujisawa et al., 2011)] in the bound regions. The frequency of PAL and N10 motifs was 0.05 to 0.23 motif/region and 0 to 0.11 motif/region, respectively, whereas the frequency of typical RIN binding motifs was 0.72 to 1.59 motif/region (Supplemental Table 5). The difference in the frequencies between PAL/N10 and typical RIN binding motifs mostly depended on the stringency of the motif sequences.

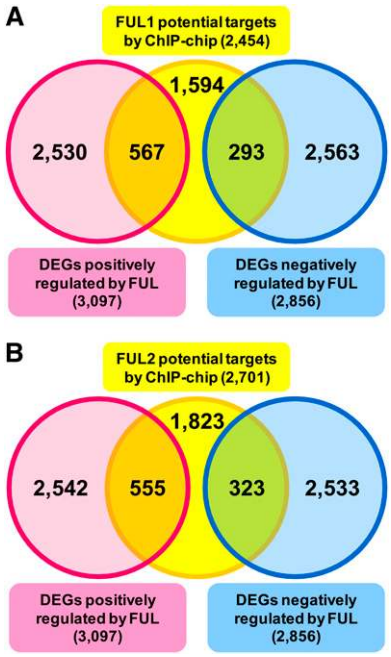

Figure 3. Identification of Direct Targets of FUL1 and FUL2 during Ripening.

Venn diagrams of potential direct target genes of FUL1 **(A)** and FUL2 **(B)** and DEGs positively and negatively regulated by FUL1 or FUL2 during ripening. DEGs positively and negatively regulated by the FUL homologs were significantly (FDR ≤ 0.05) downregulated (FC < 0.5) or upregulated (FC > 2) in the *FUL1*/*FUL2*-suppressed fruits harvested at 45 DAP compared with the same aged wild-type ripening fruits (Shima et al., 2014).

Intriguingly, the frequency of PAL sequences in the FUL2-unique regions (0.23 motif/region) was relatively higher than those in the RIN- and FUL1-unique regions (0.05 to 0.14 motif/region) compared with the other types of CArG-box motifs (Supplemental Table 5). We also searched the nonoverlapping regions for conserved sequence motifs using MEME software (Bailey et al., 2006), but we did not detect any motifs.

Large-Scale Identification of Direct FUL1 and FUL2 Target Genes

Using the genomic positions of the 2307 FUL1-bound and 2457 FUL2-bound regions, we found 2454 and 2701 genes as potential direct FUL1 and FUL2 target genes, respectively. These genes have one or more bound regions in the 2-kb promoter region or in other gene regions (exons, introns, or the 1-kb region downstream of the translation termination site) overlapping with the promoter region of a neighboring gene. To identify direct FUL1 and FUL2 target genes whose expression is actually regulated by FUL1 and FUL2 during ripening, we compared the sets of potential targets with the set of *FUL1*/*FUL2*-regulated genes (DEGs for *FUL*) identified by the RNA-Seq analysis described above. We defined the potential targets included in the DEG set as direct FUL1 or FUL2 targets. These analyses identified 860 direct targets of FUL1 (567 positively regulated and 293 negatively regulated; Figure 3A; Supplemental Data Set 5A) and 878 direct targets of FUL2 (555 positively regulated and 323 negatively regulated; Figure 3B; Supplemental Data Set 5B). The sets of direct FUL1 and FUL2 targets shared 697 genes in common (81% for FUL1 and 79% for FUL2; areas E and G in Figure 4). Using the RNA-Seq data for *rin* mutant fruits and the reanalyzed ChIP-chip data for RIN (described above), we also identified 262 direct RIN target genes, including 162 genes that were not found in the previous study (Fujisawa et al., 2013) (Supplemental Data Set 5C). Of the direct FUL1 and FUL2 targets, 215 (25%; areas D and G in Figure 4) and 210 (24%; areas F and G in Figure 4), respectively, were also direct RIN targets. Of the direct RIN targets, 217 (83%) were also direct targets of FUL1, FUL2, or both. All the sets of direct FUL1, FUL2, and RIN targets shared 208 genes (area G in Figure 4). Of the FUL1 and FUL2 target genes, 645 (75%; areas B and E in Figure 4) and 668 (76%; areas C and E in Figure 4), respectively, were identified as not belonging to the set of direct RIN target genes. Finally, we identified unique targets of each transcription factor and found 156 (18%; area B in Figure 4) genes for FUL1, 179 (20%; area C in Figure 4) genes for FUL2, and 45 (17%; area A in Figure 4) genes for RIN (Supplemental Data Sets 5A to 5C).

To test the significance of the overlap between potential direct target genes based on ChIP-chip and DEGs based on RNA-Seq, we estimated the number of overlapping genes that we would expect to occur by chance, using the formula: $Eg = Po \times De/Ge$, where *Eg* is the expected number of genes overlapping by chance, *Po* is the number of potential targets, *De* is the number of DEGs, and *Ge* is the number of all genes in the genome. For FUL1, we identified significantly more direct target genes (860) than expected by chance (*Eg* = 408, $P < 0.001$ by χ^2 test). Similarly, for FUL2 and RIN, we also identified more direct target genes (878 and 262) than we expected by chance (*Eg* = 449, $P < 0.001$ for FUL2; *Eg* = 152, $P < 0.001$ for RIN).

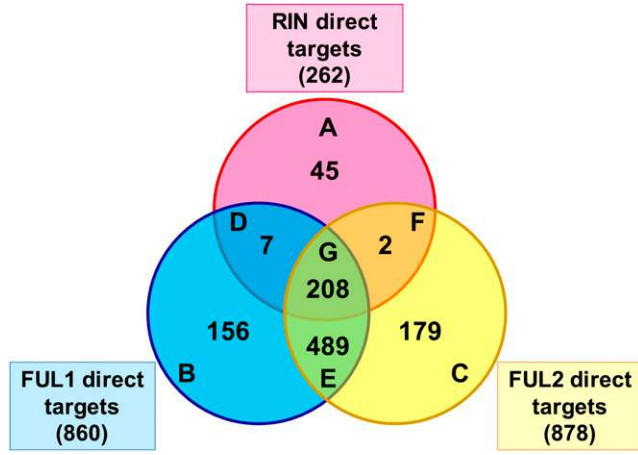

Figure 4. A Venn Diagram of Direct Targets of RIN, FUL1, and FUL2.

Of the DEGs for *rin* or *FUL1*/*FUL2*, genes that contain one or more binding regions in the promoters or other gene regions were identified as direct targets for the respective transcription factors (see text). The set of direct RIN targets includes the genes identified by reanalysis in this study.

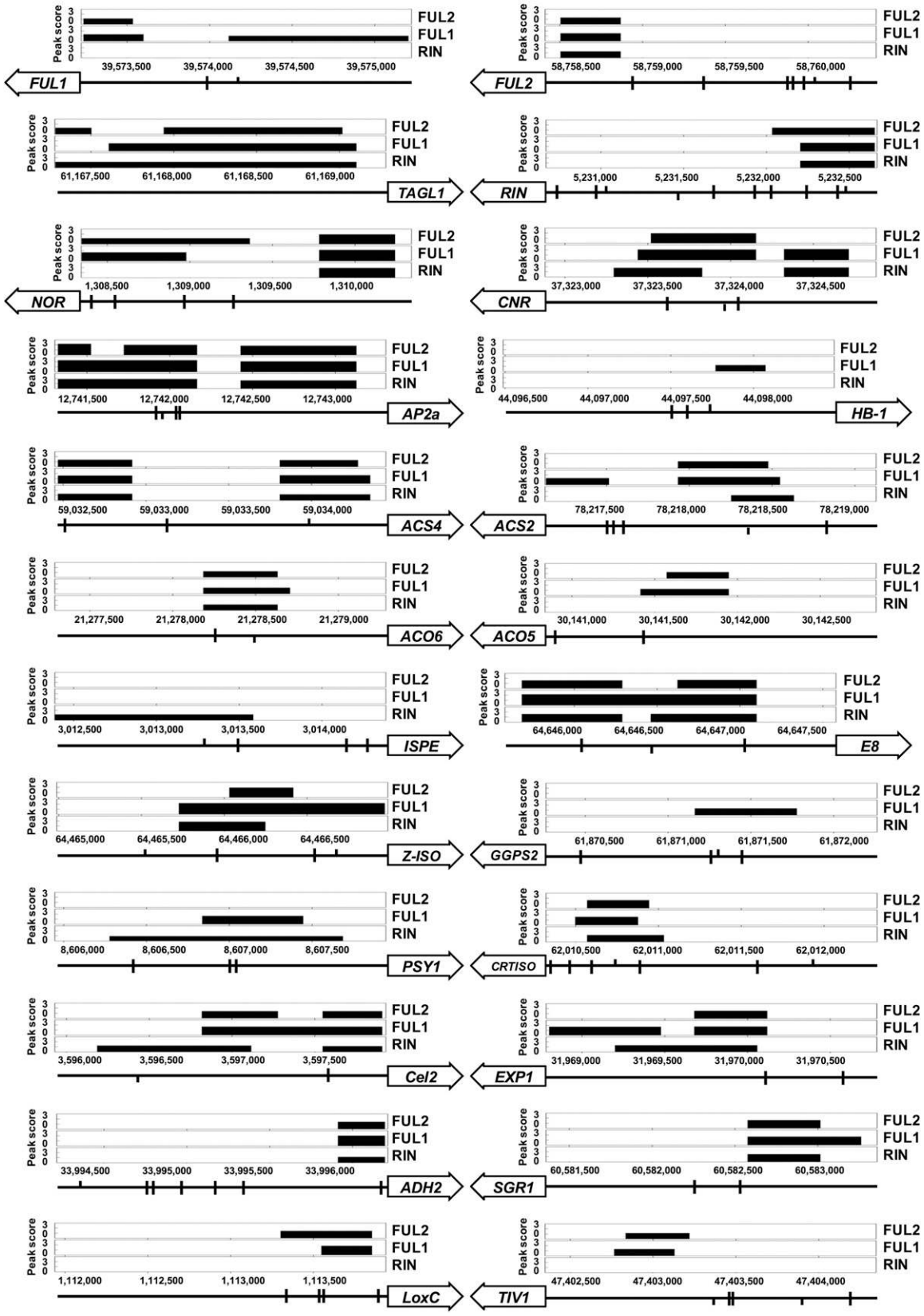

Figure 5. Examples of FUL1- and FUL2-Bound Regions Detected by ChIP-chip in the Promoters of Ripening-Related Genes.

Detection of Known Ripening-Related Genes among Direct FUL1 and FUL2 Targets

The combined ChIP-chip and RNA-Seq analysis showed that the FUL1 and FUL2 direct targets include known ripening-related direct RIN target genes, such as genes for 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (*ACS2* and *ACS4*), for an ACC oxidase homolog (*E8*), for *APETALA2a*, *NOR*, and *RIN* (Figure 5; Supplemental Data Sets 5A and 5B). The bound regions of FUL1, FUL2, and RIN overlapped with each other in the promoters of many ripening-related target genes (Figure 5). The binding of FUL1, FUL2, and RIN to the same regions strongly suggests that they regulate these target genes as a complex. However, this simple model did not apply to *TAGL1*, which has overlapping FUL1-, FUL2-, and RIN-bound regions in the promoter and was included in the sets of positive direct FUL1 and FUL2 targets, but not in the set of direct RIN targets (Figure 5; Supplemental Data Sets 5A to 5C) because the *rin* mutation showed little effect on *TAGL1* expression.

By contrast, a few ripening-associated genes had bound regions for only one of the transcription factors. For example, the promoters of genes for HD-zip homeobox protein 1 and for geranylgeranyl diphosphate synthase contain only a FUL1-bound region; genes for 4-diphosphocytidyl-2-*C*-methyl-D-erythritol kinase and for 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase contain only a RIN-bound region (Figure 5; Supplemental Data Sets 5A to 5C). We did not find any ripening-associated genes that contain only a FUL2-bound region. A gene for phytoene synthase (*PSY1*), a direct target of RIN (Martel et al., 2011; Zhong et al., 2013) and FUL1 (Shima et al., 2013), contains regions bound by FUL1 and RIN, but not FUL2, in the promoter. Binding regions both of FUL1 and FUL2, but not RIN, were detected in the promoters of *FUL1*, a gene for acid-β-fructofuranosidase (invertase; *TIV1*) and the genes for carotene β-hydroxylase (*BCH*) and 9-*cis*-epoxycarotenoid dioxygenase (*NCED*), which act in carotene conversion and in cleavage of 9-*cis* xanthophylls, respectively (Figure 5; Supplemental Data Sets 5A to 5C; as described below). These results indicated that these genes are regulated by FUL homologs in a RIN-independent manner.

By contrast, direct RIN targets, such as genes for polygalacturonase (*PG2A*), for Met sulfoxide reductase (*E4*) and *CNR*, were not included in the sets of direct FUL1 and FUL2 targets because they showed either insufficiently significant changes in expression or no detected bound region, as described previously (Fujisawa et al., 2013).

Functional Classification of Direct FUL1 and FUL2 Targets

To elucidate biological processes and pathways that FUL1 and FUL2 directly regulate during ripening, we used the MIPS classification profiles to classify the functions of direct FUL1 and FUL2 target genes. FUL1 and FUL2 targets are implicated in a wide range of biological processes, including metabolic and signaling pathways essential for fruit ripening (Supplemental Data Sets 6A and 6B). Supplemental Table 6 shows overrepresented categories with a significantly higher frequency of genes ($P < 0.001$ by Fisher's exact test) in the set of direct targets than in the whole genome. Of the overrepresented categories in the sets of positive direct FUL1 and FUL2 targets, categories related to ethylene synthesis such as "metabolism of methionine" and related to responses to various external stimuli were also enriched in the set of positive direct RIN targets (Supplemental Table 6 and Supplemental Data Set 6C). Similarly, a category related to lycopene accumulation such as "tetraterpenes (carotenoids) metabolism" was also enriched in the sets of positive direct FUL1, FUL2, and RIN targets. However, we found a difference in the distribution of their targets in the carotenoid pathway and its upstream pathways. We detected direct FUL1 targets in the pathway downstream from GGDP synthesis and direct FUL2 targets in the pathway downstream of phytoene, but direct RIN targets were limited to the pathway upstream of carotenes except for the gene encoding zeaxanthin epoxidase (*ZEP*) (Figure 6). Together, the results suggest that FUL1 and FUL2 regulate genes involved in ethylene production or response to external stimuli in collaboration with RIN, but they regulate carotenoid metabolism partly independently of RIN.

Interaction of FUL1, FUL2, RIN, and TAGL1

FUL1 and FUL2 can form heterodimers with RIN (Leseberg et al., 2008; Bemer et al., 2012; Shima et al., 2013). This is consistent with our ChIP-chip results showing that RIN and FUL homologs share binding regions on the promoters of many genes, indicating that they regulate common targets as a complex. In addition, a yeast two-hybrid screen detected interaction of RIN with a MADS box ripening regulator, TAGL1 (Martel et al., 2011). *FUL1*/*FUL2* suppression and *TAGL1* suppression resulted in similar phenotypes, including defective ripening with fruit softening (firmness was reduced similar to the untransformed control) (Itkin et al., 2009; Vrebalov et al., 2009; Giménez et al., 2010; Bemer et al., 2012; Shima et al., 2014), suggesting a functional interaction between the FUL homologs and TAGL1. In this study, to clarify complex formation of ripening-related MADS box proteins in more detail, we examined the interaction of RIN and FUL homologs with TAGL1 by in vitro gel retardation assay using CArG-box-containing DNA fragments as probes.

The gel retardation assay showed that TAGL1 was able to form DNA binding complexes with RIN and FUL2 (Figure 7A). By contrast, the formation of a DNA binding complex of FUL1 with TAGL1 was not confirmed (Figure 7A). However, if RIN was present in the synthesis mixture with TAGL1 and FUL1, the gel retardation assay detected two retarded bands (Figure 7B, lane

Figure 5. (continued).

For comparison, RIN-bound regions identified in this study are also indicated. Genomic position and $\log_2$-scale peak score of each bound region is indicated above the 2-kb gene promoters (horizontal bars). Boxed arrows with gene symbols indicate the orientation of genes. Thin vertical lines indicate the positions of CArG-boxes in the promoter. Abbreviations are given in Figure 1.

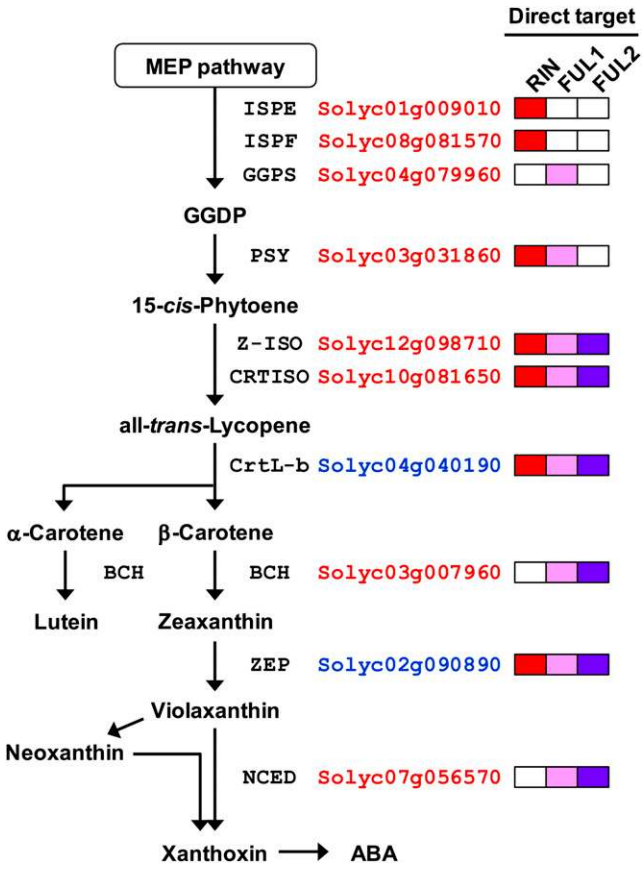

Figure 6. Direct Target Genes of RIN, FUL1, and FUL2 in Carotenoid Synthesis and Metabolism.

Only enzymes encoded by the direct target genes are shown, with gene identifiers and tags for RIN-, FUL1-, and FUL2-targeting profiles. Gene identifiers (Solyc numbers) in red and blue indicate positively and negatively regulated genes during ripening, respectively. ABA, abscisic acid; BCH, carotene β-hydroxylase; CRTISO, carotene isomerase; CrtL-b, β-lycopene cyclase; GGDP, geranylgeranyl diphosphate; GGPS, geranylgeranyl diphosphate synthase; ISPE, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol kinase; ISPF, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; MEP, methylerythritol phosphate; NCED, 9-*cis*-epoxycarotenoid dioxygenase; PSY, phytoene synthase; ZEP, zeaxanthin epoxidase.

3): a lower unknown band in addition to the upper band at the same size as RIN-TAGL1 (Figure 7B, lane 2). When the amount of FUL1-expressing vector was decreased in the in vitro protein synthesis reaction, the signal intensity of the lower band decreased, but the intensity of the upper band increased (Figure 7C). Taking these observations together, we consider that the upper and lower bands represent DNA binding of RIN-TAGL1 and FUL1-RIN-TAGL1 complexes, respectively.

FUL2, like FUL1, formed a complex with RIN and TAGL1, namely, FUL2-RIN-TAGL1 (Figure 7B, lane 5). In addition, unlike FUL1, the assay detected DNA binding of FUL2 homodimer and FUL2-TAGL1 heterodimer with a difference in DNA sequence preference: FUL2 homodimer could bind to the N10-type CArG-box but not to the PAL type, whereas FUL2-TAGL1 could bind to both types (Figure 7A). Moreover, FUL2-TAGL1 predominantly formed in the presence of FUL2 and TAGL1 rather than FUL2 homodimer, implying that the FUL2-TAGL1 heterodimer is the main form of the functional complex when they act in a RIN-independent manner.

FUL Homologs Affect Flavonoid Accumulation in Peel

A previous study on fleshy fruits found the association of a bilberry FUL homolog with flavonoid biosynthesis (Jaakola et al., 2010). To examine the association of tomato FUL homologs with flavonoid biosynthesis, we measured the accumulation of naringenin chalcone, a major flavonoid in tomato (Muir et al., 2001), in the peel of the fruits of three *FUL1*/*FUL2*-suppressed lines (TF2, TF18, and TF68) (Shima et al., 2014). We found that at P age, the fruits of all three lines had markedly less naringenin chalcone than the wild-type fruits, although the *rin* mutant fruits accumulated naringenin chalcone at similar levels to the wild-type fruits (Supplemental Figure 4A). Furthermore, to examine the effect of *FUL1*/*FUL2* suppression on gene expression, we compared RNA-Seq data among the fruits of the wild type, TF18, and *rin* mutants, for genes involved in the flavonoid pathway (Supplemental Figure 4B). The analysis showed that *FUL1*/*FUL2* suppression caused changes in expression of genes related to the flavonoid pathway compared with the wild type and *rin* mutants (Supplemental Figure 4C). Both of the positive sets of direct FUL1 and FUL2 targets included Solyc06g082540, which encodes cinnamate 4-hydroxylase, which is required for naringenin chalcone synthesis. By contrast, both of the negative sets of direct FUL1 and FUL2 targets included Solyc05g010320, which encodes a chalcone-flavonone isomerase that metabolizes naringenin chalcone (Supplemental Data Sets 5A to 5C). These two genes were not included in the set of direct RIN targets. These results suggest that the FUL homologs affect flavonoid synthesis via a RIN-independent regulatory mechanism.

DISCUSSION

Several types of transcription factors regulate tomato fruit ripening, and the MADS box factors RIN and TAGL1 act upstream of ethylene-dependent and -independent pathways. Recent studies cosuppressing *FUL1* and *FUL2* revealed that the FUL homologs also act as key regulators of many ripening processes, including lycopene accumulation (Bemer et al., 2012; Shima et al., 2014) and ripening-associated ethylene production (Shima et al., 2014). In this study, we identified hundreds of direct FUL1 and FUL2 target genes expressed during ripening and confirmed the formation of higher order complexes with FUL1, FUL2, RIN, and TAGL1, revealing a mode of regulation by these MADS box factors.

FUL Homologs Have More Diverse Roles in Ripening Than RIN

In this study, using RNA-Seq, we demonstrated the effect of *FUL1*/*FUL2* suppression on gene expression in ripening tomato fruits, drastically expanding our knowledge of the role of these *FUL* homologs. The RNA-Seq analysis detected 5953 DEGs in

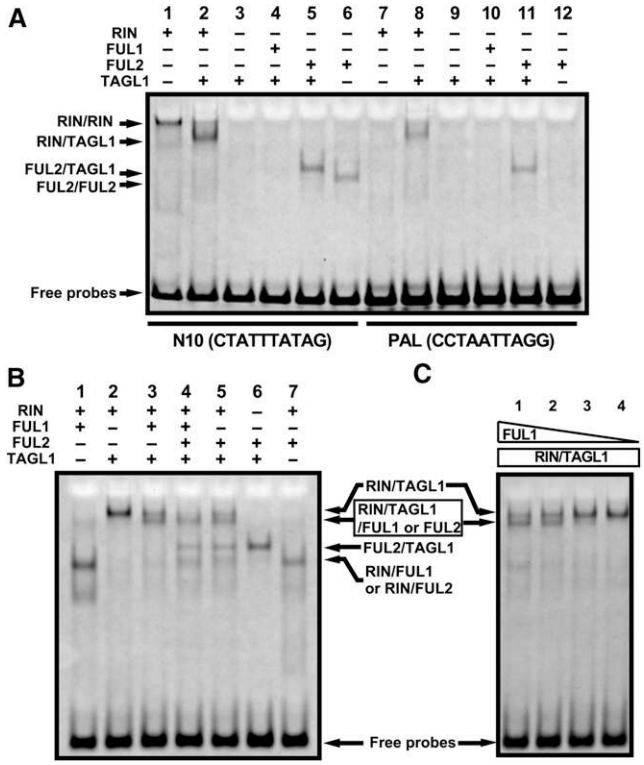

Figure 7. Gel Retardation Assays for DNA Binding Activity of the MADS Box Proteins Regulating Fruit Ripening.

(A) DNA binding complex formation between RIN, FUL1, FUL2, and TAGL1. For probe DNAs, two types of CArG motif sequences (N10 and PAL; West et al., 1998) were used.

(B) Higher order DNA binding complex formation by RIN, FUL1, FUL2, and TAGL1.

(C) Verification of the participation of FUL1 in complex formation with RIN and TAGL1. For each protein synthesis reaction, the FUL1 expression vector was added in decreasing amounts (for the lane 2 experiment, two-thirds of the vector in lane 1, for lane 3, one-third, and for lane 4, no vector), while the plasmids for RIN and TAGL1 were added equally in each experiment. In the experiments in **(B)** and **(C)**, N10 was used as the probe.

the *FUL1*/*FUL2* suppressed fruits (Shima et al., 2014), many more than a previous *FUL* RNAi analysis, which identified 895 genes (Bemer et al., 2012). This difference may be due to our RNAi construct, which may have suppressed *FUL1* and *FUL2* expression more effectively than those in the previous report (Shima et al., 2014) or the difference in cultivar (AC versus Micro-Tom) or analytical design between the two studies (RNA-Seq analysis versus microarray). The strong suppression of *FUL1*/*FUL2* may reduce the expression of their target genes enough to decrease ripening-related ethylene production. Surprisingly, the analysis indicated that the FUL homologs affect a larger number (>3000) of positively or negatively regulated genes that change during ripening (i.e., DEGs for ripening) than RIN did (Supplemental Figure 2). This and the results of MIPS classification suggest that the FUL homologs regulate a wider range of biological processes during ripening than RIN.

FUL1 and FUL2 Have Redundant and Independent Functions and Have Functions in Common with and Distinct from RIN

Our combined ChIP-chip and RNA-Seq analysis revealed that the sets of direct FUL1 and FUL2 targets include positively and negatively regulated genes, indicating the function of the FUL homologs as activators and repressors, similar to RIN (Fujisawa et al., 2013). The analysis also revealed that FUL1 and FUL2 have 697 direct target genes in common, which represent the majority (79 to 81%) of direct FUL1 and FUL2 targets. The subset of common FUL1 and FUL2 targets included many key ripening-related genes, supporting the idea of redundant functions of FUL1 and FUL2 in the regulation of ripening, as previously described (Bemer et al., 2012; Shima et al., 2014). The *FUL1* transcript was more abundant in wild type ripening than the *FUL2* transcript (P age AC fruits; Supplemental Table 7); therefore, *FUL1* may be more responsible for the redundant FUL activity during ripening than *FUL2*. The redundant FUL function may positively regulate the expression of *TAGL1*, a *SHP* homolog. This seems opposite to *Arabidopsis* carpel development, where FUL negatively regulates *SHP* (Ferrándiz et al., 2000). The existence of opposite regulatory pathways in tomato and *Arabidopsis* might reflect the distinct evolution of fleshy type fruits from dry type fruits.

FUL1 and FUL2 also have nonoverlapping target genes and bound regions, suggesting that each FUL homolog has additional independent functions; for example, "transcriptional control" was enriched only in the FUL1 target set (Supplemental Table 6). Although it is unclear to what extent these unique functions contribute to ripening, these results help clarify functional divergence between FUL1 and FUL2.

FUL1 and FUL2 may function together with and independently of RIN, as found in the carotenoid and flavonoid pathways. Of the targets of the FUL homologs in the carotenoid pathway, the genes encoding phytoene synthase 1 (*PSY1*), ζ-carotene isomerase, carotenoid isomerase, and β-lycopene cyclase are also direct RIN targets, but the genes involved in carotene conversion and its downstream processes, such as *BCH* and *NCED*, are probably not direct RIN targets (Figure 6). This clearly indicates that the FUL homologs regulate the overall pathway, but RIN is specialized to regulate lycopene accumulation, as previously described (Fujisawa et al., 2013). These findings show the divergent roles of RIN and the FUL homologs during ripening. We also clearly show that FUL homologs, but not RIN, regulate the level of a flavonoid, naringenin chalcone. This implies that the functions of FUL homologs in flavonoid biosynthesis are conserved between bilberry (Jaakola et al., 2010) and tomato.

Higher Order Complexes of MADS Box Proteins in Ripening Regulation

Our analysis revealed that 217 genes, including many key ripening-related genes, are direct targets of RIN and either FUL1 or FUL2, or both. Based on this and our previous analyses (Shima et al., 2013), we consider that RIN plays its principal role (i.e., fruit ripening regulation) as part of a DNA binding complex with the FUL homologs. Moreover, this study confirmed the RIN-TAGL1 heterodimer formation, consistent with previous studies using yeast

two-hybrid assays (Leseberg et al., 2008; Martel et al., 2011; Bemer et al., 2012; Shima et al., 2013) and higher order complex formation by RIN, FUL1/FUL2 and TAGL1. The higher order complexes may be tetramers formed by coupling RIN-TAGL1 with RIN-FUL1 or RIN-FUL2 (Supplemental Figure 5). In the tetramer, FUL1 and FUL2 can be substituted for each other, consistent with previous reports of little or no effect of single *FUL1* knockdown on fruit ripening (Jaakola et al., 2010; Bemer et al., 2012). We assume that the tetramers are mainly responsible for ripening regulation. In contrast with the common direct targets of FUL homologs and RIN, transcriptional regulation of FUL1/FUL2-independent direct RIN targets might be accomplished by RIN homodimers (Ito et al., 2008) or complexes of RIN and other factors such as TAGL1. We assume that RIN and TAGL1 form a tetramer of two RIN-TAGL1 heterodimers (Supplemental Figure 5), as observed in *Arabidopsis* where SEP3 and AGAMOUS (AG), homologs of RIN and TAGL1, respectively, form a tetramer (Smaczniak et al., 2012b).

The possible tetramer combinations may be analogous to the floral quartet model, a refinement of the classic ABC model for the *Arabidopsis* MADS domain proteins that regulate flower organ specification. In the quartet model, floral organs are specified by tetramers composed of two dimers with organ-specific combinations of four classes of MADS domain proteins (Theissen and Saedler, 2001; Smaczniak et al., 2012b). Tetramer formation by RIN, FUL1/FUL2, and TAGL1 can explain the similar ripening defects, such as inhibition of ethylene and carotenoid biosynthesis, commonly observed in the *rin* mutant (Tigchelaar et al., 1978; Vrebalov et al., 2002), the *TAGL1* knockdown lines (Itkin et al., 2009; Vrebalov et al., 2009; Giménez et al., 2010), and the *FUL1*/*FUL2*-suppressed fruits (Bemer et al., 2012; Shima et al., 2014). Furthermore, tetramer formation can also explain the observation that RIN binding to target sites occurs in a CNR-dependent manner (Martel et al., 2011) because the reduced expression of *FUL1* (*TDR4*) in *Cnr* mutant fruits (Eriksson et al., 2004) may affect tetramer formation. In MADS box complex formation, SEP proteins may function as the glue that mediates the assembly of MADS box protein complexes (Immink et al., 2009). Our results suggest that RIN, which belongs to the SEP subfamily, also conserves the assembly function to form ternary complexes (Supplemental Figure 5) similar to those assembled by *Arabidopsis* SEP3, such as FUL-SEP-SEEDSTICK (AG family) and SHP (AG family)-SEP-AG (Immink et al., 2009).

We also found that 75 to 76% of the direct FUL1 and FUL2 targets are not direct RIN targets, suggesting that the FUL homologs regulate the expression of these targets in a RIN-independent manner. Moreover, 18 to 20% of direct targets were RIN independent and exclusively targets of either FUL1 or FUL2. This means that each factor has a unique regulatory function. We consider that a FUL2-TAGL1 heterodimer may be a plausible component of the regulation of unique FUL2 targets. As shown by our gel retardation assay, FUL2 expands its DNA binding ability by interacting with TAGL1, which may allow FUL2 to recognize both N10 and PAL-type CArG-boxes, the latter of which are enriched in the FUL2 unique bound regions in the tomato genome (Supplemental Table 5). However, TAGL1 seems not to interact with FUL1 to form a DNA binding dimer complex and FUL1 is unable to form a DNA binding homodimer (Figure 7A) or heterodimer with FUL2 (Supplemental Figure 6). Thus, FUL1 may interact with unidentified partner(s) for the regulation of its unique direct targets (Supplemental Figure 5). Other possible candidates for FUL1 partners include an AG homolog (TAG1) and two SEP homologs (TM5 and TM29), based on their strong expression in ripening fruits (Supplemental Table 7). So far, however, the involvement of these candidates in fruit ripening remains unclear (Pnueli et al., 1994a, 1994b; Ampomah-Dwamena et al., 2002). Identification of a FUL1 partner will improve our understanding of transcriptional regulation of ripening and also refine the quartet model for fruit development.

METHODS

RNA-Seq

Total RNA samples were prepared from fruits of the wild type (AC cultivar), a *FUL1*/*FUL2*-suppressed line (TF18, generated by transformation of AC) (Shima et al., 2014), and *rin* mutants (a nearly isogenic line LA3754, obtained from the Tomato Genetics Resource Center [University of California, Davis]) of tomato (*Solanum lycopersicum*) at 35 and 45 DAP by SDS-phenol extraction and subsequent elimination of polysaccharides with 2-butoxyethanol (Schultz et al., 1994). The RNAs were further purified with the RNeasy Plus Kit (Qiagen). RNA quality was confirmed using a 2100 Bioanalyzer (Agilent). The RNA samples were analyzed with a HiSequation 2000 sequencer (Illumina) according to the manufacturer's instructions. qRT-PCR for verification of the RNA-Seq data was performed using Thunderbird SYBR qPCR mix (Toyobo) with a 7300 Real-Time PCR system (Applied Biosystems) as previously described (Shima et al., 2014). Primer sequences for qRT-PCR are listed in Supplemental Table 8. Three biological replicates for each stage of the lines were analyzed. MDS analysis was conducted by calculating the coefficient of variation of expression between samples for the top 500 genes that best distinguish the samples. The coefficient was calculated from normalized RNA-Seq data of *FUL1*/*FUL2*-suppressed (TF18), wild-type (AC), and *rin* mutant fruits at G and P ages (three biological replicates for each stage) using the R Bioconductor package edgeR (http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html) (Robinson et al., 2010; Robinson and Oshlack, 2010). Distances on the plot represent the coefficient values.

Detection of DEGs

Single-end RNA-Seq reads (~100 nucleotides) that passed through a filter for low-quality sequence were aligned using the Bowtie 2 program (Langmead and Salzberg, 2012) with protein coding sequences of the tomato predicted genes provided by the International Tomato Annotation Group version 2 (ITAG2; http://solgenomics.net/genomes/Solanum_lycopersicum/index.pl) (Tomato Genome Consortium, 2012). We used Bowtie 2 with the settings that allow 0 mismatches in a seed alignment to be adopted and that allow only uniquely mapped reads to be counted. Raw read counts for the genes were normalized and used for detection of DEGs between samples using the R Bioconductor package edgeR (Robinson et al., 2010; Robinson and Oshlack, 2010). The significance of FC ratio of DEGs was tested using P value from three independent samples estimated by exact test. P values were adjusted for multiple testing with the Benjamini and Hochberg method to control FDR (Benjamini and Hochberg, 1995). DEGs were selected using the following criteria: average counts per million $\geq$5 among the samples compared, and FC >2 or <0.5 with FDR $\leq$ 0.05. The raw read counts were also normalized using the reads per kilobase of protein coding sequence per million mapped reads method (Mortazavi et al., 2008) to express relative transcript levels within each sample.

ChIP-chip

ChIP-DNA from ripening tomato fruits (AC cultivar) was prepared using antibodies to FUL1 and FUL2 (Shima et al., 2013), and the subsequent hybridization analyses using Roche NimbleGen microarrays for tomato gene promoters were conducted as described previously (Fujisawa et al., 2013). Three independently prepared samples were used in separate hybridizations (including one dye-swap). Data analysis for detection of FUL1- and FUL2-bound regions was conducted as follows: The $\log_2$-transformed and normalized values of each probe in three replicates were averaged and subjected to peak detection (FDR $\leq$ 0.05) by sliding window analysis using NimbleScan software (for more detail, see Fujisawa et al., 2013). For comparison, the previously examined ChIP-chip data for RIN (Fujisawa et al., 2013) were also reanalyzed with the same algorithm. Verification of the binding by qChIP-PCR was basically conducted as previously described (Fujisawa et al., 2013) using Thunderbird SYBR qPCR mix. Primer sequences for qChIP-PCR are listed in Supplemental Table 8. Three biological replicates were analyzed.

In Silico Motif Analysis

FUZZNUC program included in the EMBOSS package (Rice et al., 2000) was used to search DNA sequences of the bound regions unique to FUL1, FUL2, and RIN for CArG-box motif sequences [PAL type, $CC(A/T)_6GG$; N10 type, $CTA(A/T)_4TAG$ (West et al., 1998); RIN binding, $C(C/T)(A/T)_6(A/G)G$ and $C(A/T)_8G$ (Ito et al., 2008; Fujisawa et al., 2011)].

Functional Gene Classification

This study used the functional gene annotation previously conducted (Fujisawa et al., 2011, 2013) for analysis of direct target genes of FUL1, FUL2, and RIN. Briefly, the tomato ITAG2 predicted gene products were tagged by *Arabidopsis thaliana* proteins based on a similarity search using BLASTP (Altschul et al., 1997). The ITAG2 genes were functionally annotated using the information for tagged *Arabidopsis* proteins in a functional catalog database provided by MIPS (http://www.helmholtz-muenchen.de/en/ibis/resourcesservices/services/funcat-the-functional-catalogue/index.html) (Ruepp et al., 2004). Significance of enrichment of genes in the subsets of direct targets relative to those in the tomato genome (the whole set of ITAG2 genes) was calculated by Fisher's exact test for each MIPS category.

Gel Retardation Assay

The expression vectors for RIN, FUL1, and FUL2 were previously constructed (Ito et al., 2008; Shima et al., 2013) and used for cell-free protein synthesis. The expression vector for TAGL1 (pEU-TAGL1) was constructed as follows: The *TAGL1* cDNA was amplified with primers specific to *TAGL1* (Supplemental Table 8), digested with *Bam*HI and *Nco*I, and inserted into the pEU-3a vector (Ito et al., 2002). Protein synthesis was performed by in vitro translation using the TnT SP6 quick-coupled transcription/translation system (Promega). Probe DNA was prepared by PCR using plasmids (pGEM-N10 and pGEM-PAL; Ito et al., 2008) that harbor a DNA fragment including the CArG-box sequence (N10, CTATTTATAG; PAL, CCTAATTAGG) (West et al., 1998) as templates with fluorescein isothiocyanate–conjugated primers, as described previously (Ito et al., 2008). Protein-DNA binding reactions were performed according to the method described previously (Riechmann et al., 1996). The reaction was electrophoresed in a 5% polyacrylamide/bisacrylamide (60:1) gel in 0.5× TBE (44.5 mM Tris-borate/1 mM EDTA), and fluorescein isothiocyanate–labeled probes were detected using a Typhoon 8600 (GE Healthcare Bio-Sciences).

Measurement of Naringenin Chalcone

The 45-DAP fruits of the wild type AC, *rin* mutant, and three *FUL*/*FUL2* suppressed lines (TF2, TF18, and TF68) were harvested, and the peel of the fruits were used for the experiment. Three biological replicates for each stage of the lines were analyzed. Naringenin chalcone accumulation in the peel was measured by liquid chromatography–tandem mass spectrometry analysis according to the method described (Iijima et al., 2008), except for a modification of the internal standard concentration (1 μg/mL of formononetin). An Agilent 1100 system (Agilent) coupled to a 4000QTRAP (AB Sciex) was used for the analysis, and the data were analyzed using Analyst software (version 1.5.2; AB Sciex).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL libraries or the tomato unigene database (for genes with SGN number; http://solgenomics.net/search/transcripts/unigene) under the following accession numbers: *ACS2*, X59139; *ACS4*, M88487; *Actin*,U60482; a gene encoding clathrin adaptor complexes medium subunit (*CAC*), SGN-U314153; *Cel2*, U13055; *CNR*, DQ672601; *FUL1*, SGN-U578128; *FUL2*, SGN-U581535; GRAS gene (Solyc07g052960), AK327648; *PSY1*, EF157835; *PG2A*, X14074; *RIN*, AF448522; *rin*, AF448523; and *TAGL1*, AY098735. The ITAG2 identifiers for tomato genes from this article are shown in Supplemental Data Sets 1, 2, 5, and 6. The RNA-Seq and ChIP-chip data are MIAME compliant and have been deposited in a MIAME-compliant database (Gene Expression Omnibus accession numbers GSE49289 and GSE49125, respectively) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/). Supplemental Data Sets 1 to 6 are deposited in the DRYAD repository: http://dx.doi.org/10.5061/dryad.4f7n5.

Supplemental Data

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

Supplemental Figure 1. Verification of RNA-Seq Data by qRT-PCR.

Supplemental Figure 2. Venn Diagram of DEGs in Tomato Fruit Ripening, in a *rin* Mutant and a *FUL1*/*FUL2*-Suppressed Line.

Supplemental Figure 3. Validation of Enrichment of ChIP-chip FUL1- and FUL2-Bound Regions by qChIP-PCR.

Supplemental Figure 4. The Effect of *FUL1/2* Suppression and the *rin* Mutation and on Expression of Genes Involved in Flavonoid Biosynthesis in Tomato Fruits.

Supplemental Figure 5. Possible MADS Box Protein Complexes of FUL1, FUL2, RIN, and TAGL1 and Their Target Genes during Ripening.

Supplemental Figure 6. A Gel Retardation Assay for FUL1, FUL2, and RIN.

Supplemental Table 1. Statistics of RNA-Seq Data from Tomato Fruits of the Wild Type, the *rin* Mutant, and the *FUL1*/*FUL2*-Suppressed Line.

Supplemental Table 2. Statistics of the ChIP-chip Results of FUL1 and FUL2.

Supplemental Table 3. Overlap of FUL1-, FUL2-, and RIN-Bound Regions Detected by ChIP-chip.

Supplemental Table 4. Analysis of Uniquely Bound Regions by FUL1, FUL2, and RIN with Each Replicate of the Other Factors.

Supplemental Table 5. Frequency of CArG-Box Sequences in Binding Regions Unique to FUL1, FUL2, and RIN.

Supplemental Table 6. Details of the Overrepresented MIPS Categories in the Direct Target Genes of FUL1, FUL2, and RIN.

Supplemental Table 7. RNA-Seq Profile of Tomato MADS Box Genes Substantially Expressed in the Ripening Fruits.

Supplemental Table 8. A List of Primers Used in This Study.

Supplemental References.

Supplemental Data Set 1. RNA-Seq Analysis of *FUL1*/*FUL2*-Suppressed and *rin* Mutant Tomato Fruits.

Supplemental Data Set 2. A List of DEGs in the *FUL1*/*FUL2*-Suppressed Fruits, the Wild-Type Ripening Fruits, and the *rin* Mutant Fruits with MIPS Annotation.

Supplemental Data Set 3. MIPS Categories Significantly Overrepresented in the Sets of DEGs for *FUL* and *rin*.

Supplemental Data Set 4. Binding regions of FUL1, FUL2, and RIN Detected by ChIP-chip and CArG-Boxes in the Regions in This Study.

Supplemental Data Set 5. A List of Direct Target Genes of FUL1, FUL2, and RIN Target Genes Identified in This Study.

Supplemental Data Set 6. Functional Annotation of Direct Target Genes of FUL1, FUL2, and RIN Target Genes.

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AUTHOR CONTRIBUTIONS

M.F., M.K., T.K., and Y.I. designed the experiments. M.F., T.N., Y.S., M.K., J.K., and Y.I. prepared samples. M.F., T.N., Y.S., and H.N. performed research. M.F. and Y.I. analyzed the data and wrote the article.

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