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WRINKLED Transcription Factors Orchestrate Tissue-Specific Regulation of Fatty Acid Biosynthesis in *Arabidopsis*[W]

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Acyl lipids are essential constituents of all cells, but acyl chain requirements vary greatly and depend on the cell type considered. This implies a tight regulation of fatty acid production so that supply fits demand. Isolation of the *Arabidopsis thaliana* WRINKLED1 (WRI1) transcription factor established the importance of transcriptional regulation for modulating the rate of acyl chain production. Here, we report the isolation of two additional regulators of the fatty acid biosynthetic pathway, WRI3 and WRI4, which are closely related to WRI1 and belong to the APETALA2–ethylene-responsive element binding protein family of transcription factors. These three WRIs define a family of regulators capable of triggering sustained rates of acyl chain synthesis. However, expression patterns of the three *WRIs* differ markedly. Whereas only WRI1 activates fatty acid biosynthesis in seeds for triacylglycerol production, the three WRIs are required in floral tissues to provide acyl chains for cutin biosynthesis and prevent adherence of these developing organs and subsequent semisterility. The targets of these WRIs encode enzymes providing precursors (acyl chain and glycerol backbones) for various lipid biosynthetic pathways, but not the subsequent lipid-assembling enzymes. These results provide insights into the developmental regulation of fatty acid production in plants.

INTRODUCTION

Acyl lipids, which are derived from fatty acids, are essential constituents of all plant cells, where they have different functions. First, they are basic components used for membrane biosynthesis and repair. Second, triacylglycerols (TAGs; triesters of fatty acids and glycerol) are the major form of carbon and energy storage in the seeds and fruits of several plant species (Baud and Lepiniec, 2010). Third, minor amounts of plant lipids and their metabolic derivatives, like jasmonate, participate in signaling pathways (Wasternack, 2007). Finally, cuticular lipids (cuticular waxes and cutin) coating the surface of epidermal cells serve as a vital hydrophobic barrier preventing water loss, entry of pathogenic microorganisms, and organ adherence (Kunst and Samuels, 2009). Even though the structures and properties of these different acyl lipids vary greatly, they are all derived from the same fatty acid and glycerolipid biosynthesis pathway (Harwood, 1996). The fatty acid biosynthesis pathway is found in the plastids of every plant cell. Briefly, the pyruvate dehydrogenase complex generates acetyl-CoA, the building block used for fatty acid production. Fatty acid biosynthesis begins with the formation of malonyl-CoA from acetyl-CoA by heteromeric acetyl-CoA carboxylase. The malonyl group of malonyl-CoA is then transferred to an acyl carrier protein (ACP) by a malonyl-CoA:acyl carrier protein malonyltransferase. Acyl chains are produced by the fatty acid synthase complex, which uses acetyl-CoA as a starting unit while malonyl-ACP provides the two-carbon units required for chain elongation. Acyl chains are ultimately hydrolyzed by acyl-ACP thioesterases that release fatty acids.

Whereas mesophyll cells contain 5 to 10% acyl lipids by dry weight, mostly in the form of membrane lipids (Ohlrogge and Browse, 1995), some cells in the fruit mesocarp or in the seed of some plant species can accumulate up to 90% TAGs by dry weight (Bourgis et al., 2011). These observations strongly suggest that the rate of acyl chain production can vary greatly and is tightly regulated, allowing the balancing of carbon supply and demand for acyl chains to meet the requirements of a given cell type (Ohlrogge and Jaworski, 1997). Extensive transcriptomic analyses of *Arabidopsis thaliana* and other plant species have shown that transcript levels of genes encoding core fatty acid biosynthetic enzymes change in a coordinated and proportional manner to the rates of acyl chain production in the tissues analyzed (Baud and Lepiniec, 2009; Barthole et al., 2012). These analyses point out the key role played by transcriptional regulation in the control of fatty acid biosynthesis. They also suggest that the expression of most

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fatty acid biosynthetic genes might be coregulated. A large part of our current knowledge regarding these regulations has been generated from the analysis of *Arabidopsis* mutants affected in seed oil content (Focks and Benning, 1998). Whereas early developing seeds exhibit low glycerolipid contents similar to that of foliar tissues, the rate of acyl chain production dramatically increases in maturing seeds as TAGs are massively synthesized and stored in the embryo. The WRINKLED1 (WRI1) transcription factor triggers the concomitant upregulation of genes involved in fatty acid production at the onset of the seed maturation phase (Cernac and Benning, 2004). This member of the APETALA2-ethylene-responsive element binding protein (AP2-EREBP) family binds to promoter sequences of late glycolytic and fatty acid biosynthetic genes (Baud et al., 2007b). A nucleotide sequence [CnTnG(n)$_7$CG] conserved among proximal upstream regions of several target genes and called the AW-box was proposed to be recognized by WRI1 (Maeo et al., 2009). Recent studies performed in maize (*Zea mays*) and other plant species suggest that these *trans*- and *cis*-regulatory elements might be conserved between dicots and monocots (Shen et al., 2010).

In *wri1* mutant lines, the lack of transcriptional activation of the fatty acid biosynthetic pathway in early maturing embryos is responsible for a severe defect in TAG biosynthesis and results in the production of wrinkled seeds depleted in oil (Focks and Benning, 1998). However, *wri1* knockout mutants maintain a basal level of late glycolytic and fatty acid biosynthetic gene transcriptional activity, so that their vegetative development is not compromised (Baud et al., 2009). This observation, among others, indicates that the transcriptional machinery regulating the fatty acid biosynthetic pathway is probably composed of multiple regulatory proteins. Partners of WRI1, functional homologs, or even transcription factors belonging to redundant regulatory pathways might participate in the control of acyl chain production.

To isolate these unknown regulators, complementary screening procedures have been undertaken. In this article, we report the isolation and functional characterization of WRI3 (At1g16060) and WRI4 (At1g79700). Like WRI1, they belong to the AP2-EREBP family of transcription factors, and they behave as transcriptional activators of the fatty acid biosynthetic pathway. However *WRI1*, *WRI3*, and *WRI4* are clearly differentially expressed, and comprehensive characterization of corresponding single, double, and triple mutants further established that the three transcription factors do not have identical functions in planta. Whereas only WRI1 triggers sustained rates of fatty acid production in TAG-storing seeds, the three WRIs redundantly stimulate acyl chain production in developing flowers, providing precursors for cutin production and thus preventing organ adherence and subsequent semi-sterility. Taken together, these results establish another physiological consequence of deficiency in *WRI1*-like genes and provide original insights into our understanding of the developmental regulation of fatty acid production in plants.

RESULTS

Isolation of New *ProBCCP2* Binding Proteins

To identify new transcriptional regulators of the fatty acid biosynthetic pathway, a yeast one-hybrid screen was conducted with a 180-bp promoter fragment of *BIOTIN CARBOXYL CARRIER PROTEIN2* (*BCCP2*), a gene encoding a subunit of heteromeric acetyl-CoA carboxylase. Previous functional analyses revealed that this fragment contained information required for the proper regulation of *BCCP2* expression in planta (Baud et al., 2009). An ordered cDNA expression library for a set of *Arabidopsis* transcription factor open reading frames (REGIA library; Paz-Ares, 2002) was screened by mating with a yeast strain containing the *ProBCCP2*:*HIS3* construct and subsequent selection on His-free medium. Among the 926 open reading frames composing the library, three candidates were thus isolated. One of the candidates encoded WRI3, a member of the AP2-EREBP family closely related to WRI1 and belonging to the WRI1-like group (Figure 1A). Since two members of this group, namely, WRI1 and WRI4 (At1g79700), were absent from the REGIA library, the four full-length cDNAs (*WRI1*, *WRI2*/At2g41710, *WRI3*, and *WRI4*) were systematically cloned and expressed as GAL4 activating domain fusions in yeast cells. Three of the corresponding proteins, WRI1, WRI3, and WRI4, were able to induce yeast growth on selection medium, demonstrating their interaction with *ProBCCP2* (Figure 1B), whereas WRI2 did not. To further evaluate the strength of these interactions, quantitative β-galactosidase assays were performed in yeast containing the *lacZ* reporter cloned downstream of the *ProBCCP2* promoter sequence (Figure 1C). Both WRI1 and WRI4 could activate the reporter gene at high levels. The reporter activity measured in the presence of WRI3 was weaker, although significantly different from the negative controls. The reporter activity measured in the presence of WRI2 was similar to the control, thus confirming a lack of interaction with *ProBCCP2*.

WRI3 and WRI4 Are Transcriptional Activators of the Fatty Acid Biosynthetic Pathway

In order to determine whether WRI3 and WRI4, like WRI1 (Masaki et al., 2005), possess transcriptional activity, coding regions of *WRI*s were individually cloned in frame with the GAL4 DNA binding domain (GAL4-DBD). The constructs obtained were then introduced into the yeast strain AH109, which carries the *HIS3* and *ADE2* reporter genes under the control of heterologous GAL4-responsive upstream activating sequences and promoter elements. The expression of these two reporters could be activated in the presence of WRI1, WRI3, or WRI4 fused to GAL-DBD, thus establishing their transcriptional activity (Figure 2A).

To test the ability of WRI3 and WRI4 to interact with the promoter sequence of another gene involved in fatty acid production, a 950-bp promoter fragment relative to the translational start codon of *BCCP1* (At5g16390) was used as bait in a yeast one-hybrid assay (Figure 1B). The expression of WRI1, WRI4, and to a lesser extent WRI3 resulted in the specific growth of the strain on medium lacking His, thus demonstrating the interaction of WRI1 and WRI4 with *ProBCCP1*. The interaction observed with WRI3 was weaker, while no interaction could be detected with WRI2, thus confirming the results obtained with *ProBCCP2*.

To further investigate the transcriptional activation of fatty acid biosynthetic genes by the WRI transcription factors in planta, *WRI* cDNAs were cloned downstream of the strong and seed-specific *AT2S2* (At4g27150) promoter (Pouvreau et al.,

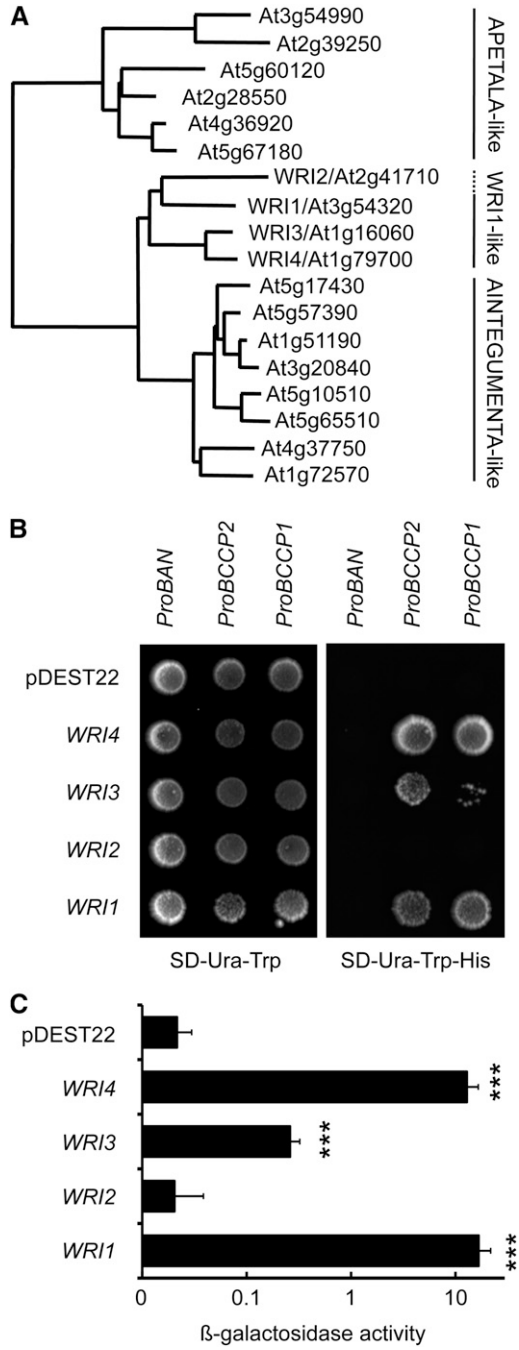

Figure 1. Interaction of the WRI Transcription Factors with Promoters of the *BCCP* Genes in Yeast.

(A) Phylogram, with branch lengths in arbitrary units, using the alignment generated by the MAFFT program. Sequences of the double AP2 (DNA binding) domains of the transcription factors (with gaps) were used for the distance analyses. A version of this phylogram including statistical support for the nodes is available as Supplemental Figure 9 online.

(B) Analysis of WRI binding to the promoters of *BCCP2* and *BCCP1* in yeast one-hybrid experiments. Yeast strains containing the *HIS3* reporter gene under the control of either the *BANYULS* (*BAN*; negative control), *BCCP2*, or *BCCP1* promoter were transformed with either the empty pDEST22 expression vector or with a version of this vector allowing the expression of WRI1, WRI2, WRI3, or WRI4 before being plated on appropriate media to maintain the expression of the vectors (SD-Ura-Trp) and to test the activation of the *HIS3* reporter gene (SD-Ura-Trp-His). Data presented are representative from the results obtained for five independent colonies.

(C) Quantification of WRI binding to the promoter of *BCCP2* in yeast one-hybrid experiments. Yeast strains containing the *LacZi* reporter gene under the control of the *BCCP2* promoter were transformed with either the empty pDEST22 expression vector or with a version of this vector allowing the expression of WRI1, WRI2, WRI3, or WRI4 before being plated on appropriate media to maintain the expression of the vectors. β-Galactosidase activity was then measured to test the activation of the *LacZi* reporter gene. Values are the means and SE of between five and six measurements performed on independent colonies. ***Significant difference according to *t* test, $P < 0.001$.

2011). The constructs thus obtained were introduced into a *wri1-4* mutant background. Overaccumulation of *WRI* RNA in the transgenic lines was verified by quantitative RT-PCR experiments performed on cDNAs prepared from siliques aged 16 d after anthesis (DAA) (see Supplemental Figure 1 online). To check whether the overexpression of WRI transcription factors could result in an efficient transcriptional activation of fatty acid biosynthetic genes, relative mRNA levels of two of these genes, *BCCP2* and *PYRUVATE DEHYDROGENASE* (*PDH*)*-E1a* (At1g01090), were quantified in this material (Figure 2B). RNA accumulation for these two genes was strongly induced in *ProAT2S2*:*WRI1*, *ProAT2S2*:*WRI3*, and *ProAT2S2*:*WRI4* lines. In order to determine whether the WRI transcription factors were able to activate the whole fatty acid biosynthetic pathway, the constructs were tested for their ability to complement the seed phenotype of the *wri1-4* mutant (Baud et al., 2007b). Microscopy observations of mature dry seeds showed a reversion of the wrinkled phenotype of *wri1-4* seeds expressing *WRI1*, *WRI3*, or *WRI4* (Figure 2C). No phenotypic reversion was obtained with *WRI2*. Total fatty acid analyses performed on dry seeds confirmed the ability of *WRI1*, *WRI3*, and *WRI4* to efficiently activate fatty acid biosynthesis and thus to restore normal oil accumulation in *wri1-4* seeds (Figure 2D).

Members of the WRI Clade Are Differentially Expressed

The expression patterns of three WRIs that interact with promoters of fatty acid biosynthetic genes and transcriptionally activate the fatty acid biosynthetic pathway were examined. First, the spatiotemporal activities of *WRI1*, *WRI3*, and *WRI4* promoters were investigated. Promoter fragments corresponding to region −1028 to −1 bp (*WRI1*), −1000 to −1 bp (*WRI3*), and −1000 to −1 bp (*WRI4*) were individually fused translationally to the *uidA* reporter gene. The corresponding constructs were assayed for their *uidA* expression patterns in transgenic *Arabidopsis* lines. As previously reported, *ProWRI1* activity was specifically observed in maturing embryos (Figures 3A to 3E) (Baud et al., 2007b). On the contrary, *ProWRI3* and *ProWRI4* activities were not detected in embryos (Figures 3J and 3O). β-Glucuronidase (GUS) staining could be observed only in flowers of the corresponding *ProWRI3*:*uidA* (Figures 3F) and

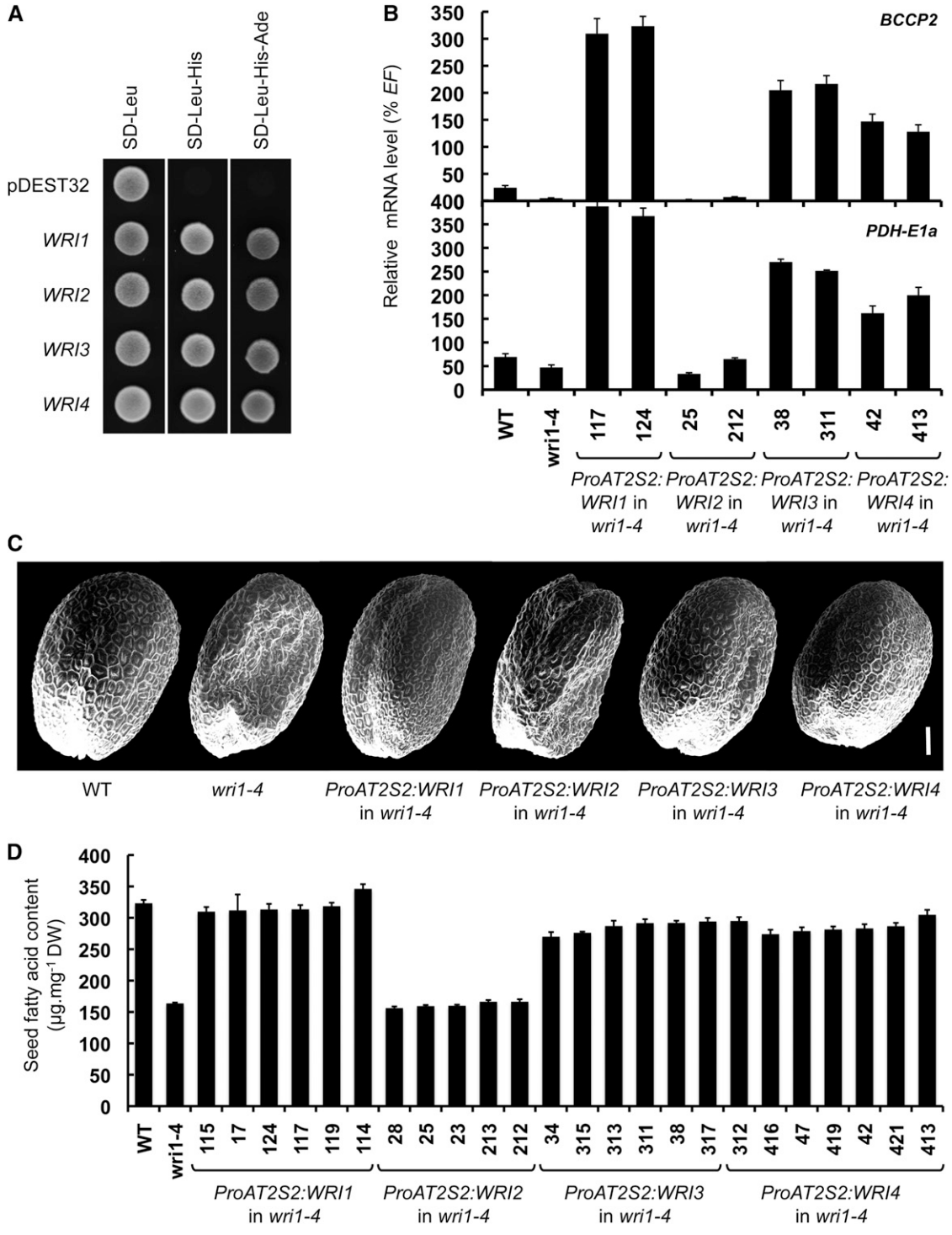

Figure 2. Transcriptional Activation of Fatty Acid Biosynthetic Genes by the WRI Transcription Factors.

(A) Transcriptional activity of the WRIs. *WRI* coding sequences were cloned in frame with GAL4-DBD, and the fusion constructs were introduced into reporter yeast containing the *HIS3* and *ADE2* reporter genes, before being plated on appropriate media to maintain the expression of the vectors (SD-Leu) and to test the activation of the *HIS3* (SD-Leu-His) or *HIS3* and *ADE2* reporter genes (SD-Leu-His-Ade). Data presented are representative from the results obtained for five independent colonies.

(B) Overexpression of fatty acid biosynthetic genes in response to seed-specific overexpression of *WRI* cDNAs. For each cDNA overexpressed, two representative transformants were analyzed. Analyses of the relative mRNA levels in siliques aged 16 DAA of two fatty acid biosynthetic genes (*BCCP2*

ProWRI4:*uidA* lines (Figures 3K). A closer examination of this material showed that pollen grains were highly stained in these lines, both before and after their release from anthers (Figures 3G to 3I and 3L to 3N). In *ProWRI4*:*uidA* lines, but not in *ProWRI3*:*uidA* lines, nectaries of the flowers were also stained (Figures 3H). To gain complementary information about the expression patterns of the *WRI*s, quantitative RT-PCR experiments were performed on cDNAs prepared from various tissues of wild-type plants (ecotype Columbia-0 [Col-0]) (Figure 3P). *WRI3* and *WRI4* mRNA accumulation patterns were clearly distinct from the *WRI1* pattern. They appeared to be ubiquitously accumulated, but at low levels, whereas high *WRI1* mRNA levels were detected in early maturing seeds. The highest *WRI3*/*WRI4* mRNA levels were measured in plant vegetative parts (e.g., stems) and in flowers.

Functions of WRI3 and WRI4 Are Not Redundant with That of WRI1 in Seeds

To investigate the function of *WRI3* and *WRI4* in planta, T-DNA insertion alleles were isolated and characterized for each gene (Figure 4A). Homozygous lines were generated, and the transcripts in each mutant line were analyzed by RT-PCR on cDNAs prepared from seeds aged 12 DAA (*WRI1* and *WRI3*) and from flowers (*WRI4*). Full-length *WRI3* transcripts were not detectable in the *wri3-1* and *wri3-2* lines. Full-length *WRI4* transcripts were absent in the *wri4-1* and *wri4-3* lines, whereas *wri4-2* mutants exhibited lower *WRI4* transcript levels when compared with the wild type. To test whether the loss of a functional *WRI* gene was compensated for by the overexpression of other family members, a quantitative RT-PCR experiment was performed on cDNAs prepared from maturing seeds of the mutant lines studied (Figure 4B). No such compensatory effect was detected for any of the mutations considered.

WRI1 is a transcriptional enhancer of the fatty acid biosynthetic pathway in maturing seeds, where high rates of fatty acid synthesis are required to sustain important TAG production (Cernac and Benning, 2004; Baud et al., 2009). To determine whether WRI3 and WRI4 participate in triggering acyl chain production in seeds, double *wri1 wri3*, *wri1 wri4*, *wri3 wri4* mutants and triple *wri1 wri3 wri4* mutants were obtained. The single, double, and triple mutants (19 genotypes) were grown under controlled conditions, and their mature dry seeds were subjected to biochemical analyses. Determinations of total fatty acid contents demonstrated that only the *wri1* mutation impacted on seed oil content (Figure 4C). To rule out the possibility of an involvement of WRI3 and WRI4 in the transcriptional activation of fatty acid biosynthesis in seeds, relative mRNA levels of two fatty acid biosynthetic genes, *BCCP2* and *PDH-E1a*, were quantified using mRNAs extracted from early maturing seeds aged 12 DAA in a subset of eight genotypes and analyzed by quantitative RT-PCR (Figure 4D). These data confirmed that *wri3* and *wri4* mutations had no effect on the transcriptional activation of fatty acid biosynthetic genes in seeds.

To further elucidate the changes leading to oil depletion observed in seeds exhibiting a *wri1* mutation, a quantitative RT-PCR approach was used to systematically analyze the relative mRNA accumulation of genes encoding enzymes involved in either fatty acid biosynthesis or TAG assembly (Figure 5). All the glycolytic and late fatty acid biosynthetic genes assayed (*PKp2*/At5g52920, *MAT*/At2g30200, *KASI*/At5g46290, *KASIII*/At1g62640, *ENR*/At2g05990, and *FATA*/At3g25110) were significantly downregulated in early-maturing seeds of the triple *wri1 wri3 wri4* mutants. Likewise, genes encoding glycerol-3-phosphate dehydrogenases (At2g41540 and At3g07690), the enzymes responsible for the provision of glycerol backbones required for TAG assembly, were repressed in mutant seeds. On the contrary, the relative mRNA levels of genes involved in TAG formation (*GPAT9*/At5g60620, *LPAAT4*/At1g75020, *DAGAT1*/At2g19450, and *PDAT1*/At3g44830) were unchanged in the seeds of mutant lines. Genes encoding prominent enzymes involved in fatty acid modification like *FATTY ACID DESATURASE2* (*FAD2*/At3g12120), *FAD3* (At2g29980), or *FATTY ACID ELONGASE1* (*FAE1*/*KCS18*/At4g34520) were unaffected by the *wri* mutations (see Supplemental Figure 2 online). *REDUCED OLEATE DESATURATION1* (*ROD1*/At3g15820), which encodes an enzyme regulating TAG composition by interconverting diacylglycerol and phosphatidylcholine during TAG biosynthesis, appeared to be downregulated in the triple mutants.

Role of the WRI Transcription Factors in the Production of Floral Cutin

While studying the *wri* lines, the 19 mutant genotypes considered were grown together with wild-type plants in a controlled environment (65% relative humidity growth chamber), and clear morphological phenotypes were observed. Whereas no visual change could be seen in these plants during vegetative growth, their reproductive organs were severely affected. The phenotypes included strong adherence between floral organs preventing flower opening (Figures 6A to 6C) and semisterility (Figure 6B). A quantification of these phenotypes (relative

Figure 2. (continued).

and *PDH-E1a*) were performed by quantitative RT-PCR. The results obtained were standardized to the constitutive *EF1αA4* gene expression level (*EF*; Nesi et al., 2000). Three independent cDNA preparations were analyzed; one or two technical replications were performed with each cDNA population analyzed. Values are the means and SE of between three and six measurements. WT, the wild type (Col-0).

(C) Rescue of the wrinkled phenotype of mature *wri1-4* seeds by seed-specific overexpression of *WRI* cDNAs. Dry seeds from *wri1-4* mutants transformed with *ProAT2S2*:*WRI* constructs were observed by scanning electron microscopy. Representative seeds are presented. Bar = 100 μm.

(D) Rescue of the total fatty acid content in mature *wri1-4* seeds by seed-specific overexpression of *WRI* cDNAs. The fatty acid contents of dry seeds from *wri1-4* mutants transformed with *ProAT2S2*:*WRI* constructs were determined by gas chromatography analysis. Values are the means and SE of five replicates performed on batches of 20 seeds from five distinct individuals. DW, dry weight.

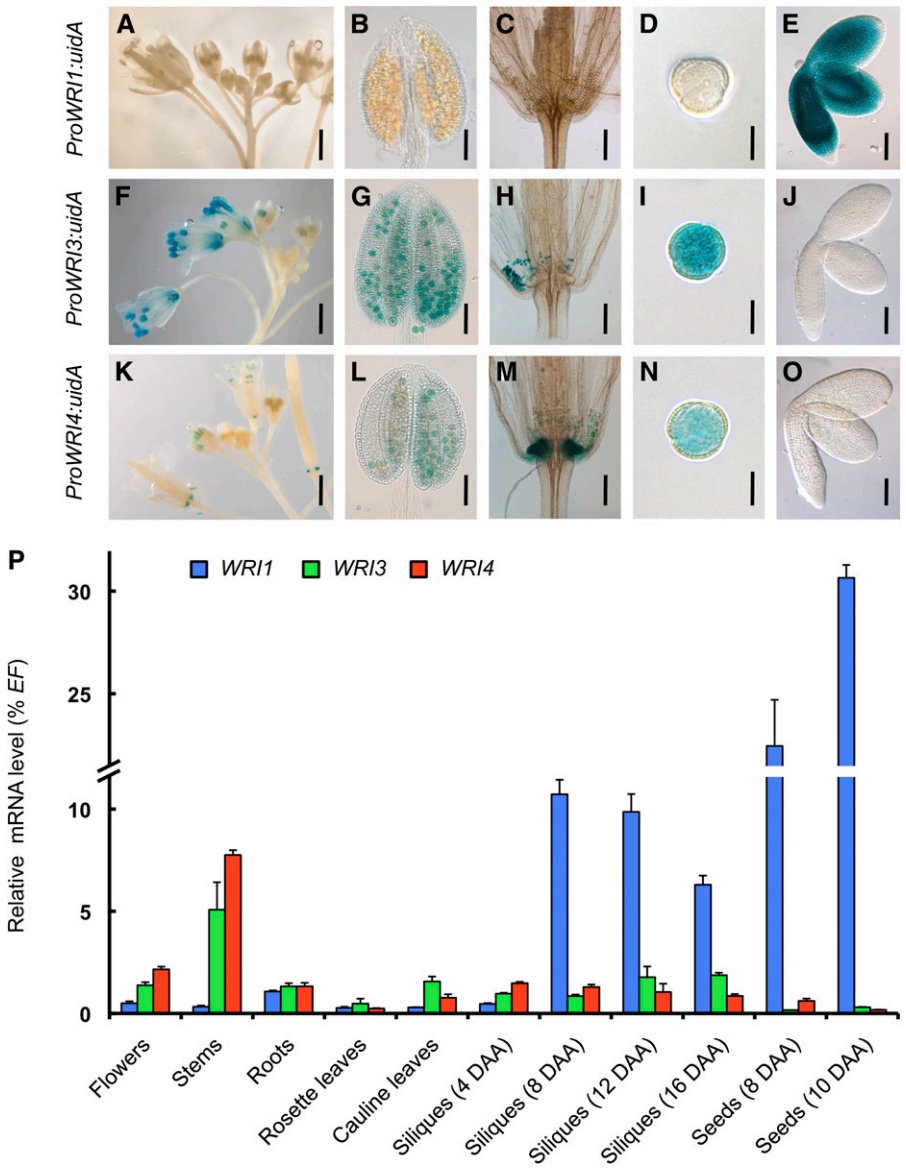

Figure 3. Expression Patterns of *WRI1*, *WRI3*, and *WRI4.*

(A) to **(O)** Pattern of activity of the *ProWRI1*:*uidA* (**[A]** to **[E]**), *ProWRI3*:*uidA* (**[F]** to **[J]**), and *ProWRI4*:*uidA* cassettes (**[K]** to **[O]**) in various plant organs. For histochemical detection of GUS activity, tissues were incubated 4 h for maturing embryos (**[E]**, **[J]**, and **[O]**) or overnight for inflorescences (**[A]**, **[F]**, and **[K]**), flowers (**[C]**, **[H]**, and **[M]**), stamens (**[B]**, **[G]**, and **[L]**), and pollen (**[D]**, **[I]**, and **[N]**) in a buffer containing 2 mM (embryos) or 0.2 mM (inflorescences, flowers, stamens, and pollen) each of potassium ferrocyanide and potassium ferricyanide. The results for GUS activity were first observed on whole-mounted inflorescences; microscopy observations of stamens, flowers, pollen grains, and excised embryos were performed using Nomarski optics. Bars = 5 mm in **(A)**, **(F)**, and **(K)**, 50 μm in **(B)**, **(G)**, and **(L)**, 200 μm in **(C)**, **(H)**, and **(M)**, 20 μm in **(D)**, **(I)**, and **(N)**, and 100 μm in **(E)**, **(J)**, and **(O)**.

(P) Analyses of *WRI1*, *WRI3*, and *WRI4* relative mRNA accumulation levels were performed in different plant organs and in developing seeds. The results obtained were standardized to the constitutive *EF1*α*A4* (*EF*) gene expression level. Values are the means and SE of three to four replicates performed on cDNA dilutions obtained from three independent mRNA extractions. DAA, days after anthesis.

proportions of unopened flowers or aborted siliques) in the 19 mutant genotypes showed that the effects of the three mutations were additive (Figure 6D; see Supplemental Figure 3A online), with the relative impact of the mutations being as follows: *wri3* > *wri4* >> *wri1*. Notably, these phenotypes were barely detectable in greenhouses with an uncontrolled humidity environment. These severe conditional phenotypes were reminiscent of those described for several mutants affected in cuticle biosynthesis (Bourdenx et al., 2011; Shi et al., 2011). Accordingly, toluidine blue surface permeability assays (Tanaka et al., 2004; Li-Beisson et al., 2009) demonstrated that the cuticle of petals (and sepals to a lesser extent) was perturbed in *wri*

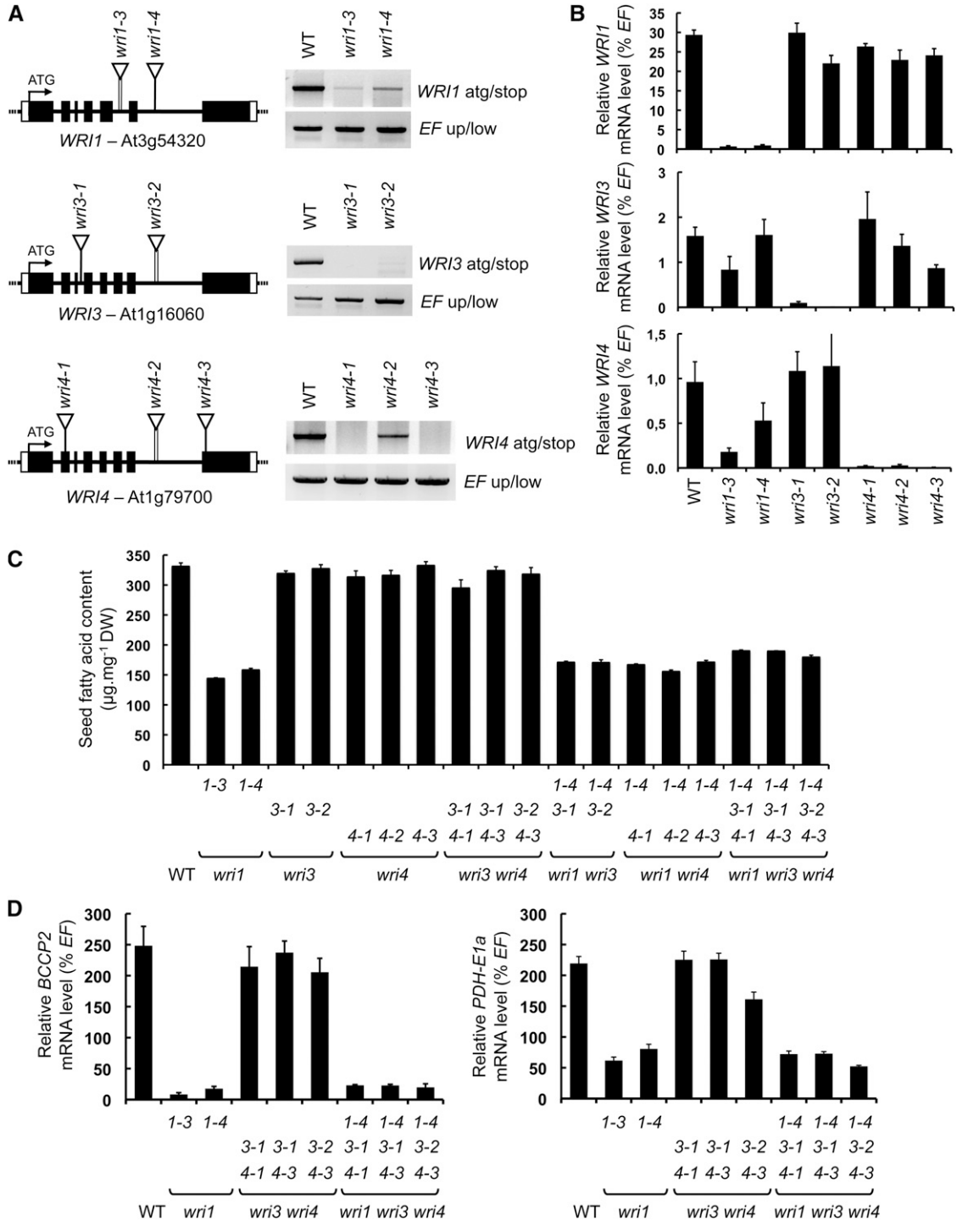

Figure 4. Characterization of *wri* Alleles.

(A) Molecular characterization of the *wri* mutations. Structures of the *WRI* genes showing the position of T-DNA insertions in *wri1-3*, *wri1-4*, *wri3-1*, *wri3-2*, *wri4-1*, *wri4-2*, and *wri4-3* are presented. For each T-DNA insertion considered, confirmed flanking sequence tag(s) are anchored in the gene structure and represented by vertical bar(s). Closed boxes represent exons, while open boxes represent untranslated regions. Accumulation of *WRI* mRNAs in wild-type and mutant backgrounds was studied by RT-PCR either in developing seeds aged 12 DAA (*WRI1* and *WRI3*) or in flowers (*WRI4*). *EF1α4* (*EF*) gene expression was used as a constitutive control. WT, the wild type (Col-0).

mutants, with these defects being particularly pronounced in the triple mutants (Figure 6D; see Supplemental Figure 3B online). To test whether increased cuticle permeability in flowers of the triple mutants was associated with changes in cutin, flowers were subjected to extensive delipidation, and the amount and composition of residual bound lipids were analyzed by gas chromatography–mass spectrometry. The accumulation of dicarboxylic acids and ω-hydroxy acids, the most abundant cutin constituents, was severely affected in the mutants (Figure 7). By contrast, the amounts of other minor constituents of cutin were not significantly different in the mutants. The total amounts of dicarboxylic and ω-hydroxy fatty acids were then determined for flowers from a subset of genotypes comprising simple, double, and triple mutants (Figure 6E). The results obtained established that the negative impacts of the *wri* mutations on cutin deposition were additive. Complementary analyses showed that cutin deposition was also altered on stems of the triple mutants, but not on rosette leaves (see Supplemental Figure 4 online). As for cuticular waxes, which are usually accumulated at lower amounts than cutin at the surface of epidermal cells, their production was not affected in the triple mutant (see Supplemental Figure 5 online).

To characterize the involvement of WRI1, WRI3, and WRI4 in the transcriptional activation of fatty acid biosynthesis in floral organs, mRNAs were extracted from petals and sepals in the same subset of eight genotypes. We first verified by quantitative RT-PCR that the loss of a functional *WRI* gene in these tissues was not compensated for by the overexpression of other family members (see Supplemental Figure 6 online). Relative *BCCP2* mRNA levels were then quantified (Figure 6F). *wri1*, *wri3*, and *wri4* mutations had additive negative effects on the transcription of this fatty acid biosynthetic gene. To further elucidate the impact of the *wri* mutations on cuticle biosynthesis, relative mRNA levels of genes involved either in acyl chain production or in cuticle biosynthesis were measured by quantitative RT-PCR experiments in petals and sepals of the triple mutants (Figure 8). Most of the glycolytic and late fatty acid biosynthetic genes assayed (*PDH-E1a*/At1g01090, *BCCP2*/At5g15530, *MAT*/At2g30200, *KASI*/At5g46290, and *ENR*/At2g05990) were significantly downregulated in the triple mutants. However, *FATA* (At3g25110) was an exception to this rule. The relative mRNA levels of genes involved in cuticle formation (*CYP86A2*/At4g00360, *CYP86A8*/At2g45970, *CYP77A6*/At3g10570, *GPAT4*/At1g01610, *GPAT6*/At2g38110, and *GPAT8*/At4g00400) were unchanged in the mutant lines.

DISCUSSION

Factors controlling the rate of fatty acid production in plants and the integration of this biochemical process into the framework of plant development have long remained unknown. The isolation and characterization of the WRI1 transcription factor provided important insights into the regulation of acyl chain production (Cernac and Benning, 2004). In particular, these studies highlighted the importance of transcriptional regulation in the coordinated activation of the fatty acid biosynthetic pathway (Baud et al., 2009). Here, we report the identification and comprehensive characterization of WRI3 and WRI4, two transcription factors involved in the regulation of fatty acid biosynthesis in *Arabidopsis*. Like WRI1, they belong to the AP2-EREPB family of transcription factors. Complementary studies performed in yeast and in planta have established that these three factors share common target genes and activate the whole fatty acid biosynthetic pathway. However, the detailed analysis of *WRI1*, *WRI3*, and *WRI4* expression patterns revealed clearly distinct expression profiles, thus suggesting different biological functions of these factors. This was confirmed by the fine characterization of a collection of single, double, and triple knockout mutants. The data obtained indicate that only WRI1 enhances the transcription of fatty acid biosynthetic genes in maturing seeds, while in developing petals and sepals, the three transcription factors are involved in the activation of fatty acid biosynthesis. In seeds, sustained rates of acyl chain production are required for massive TAG storage, whereas in floral organs, high rates of fatty acid production are essential for cutin production. These analyses establish another physiological consequence of deficiency in *WRI1*-like genes and allow us to demonstrate the importance of these WRI transcription factors in the provision of acyl chains for the biosynthesis of different classes of complex lipids. Taken together, these data allow a better understanding of how fatty acid biosynthesis can be developmentally regulated to answer various cellular demands with respect to acyl chain supply.

New Transcriptional Regulators of the Fatty Acid Biosynthetic Pathway

The transcription factor WRI1 was the first positive regulator of fatty acid biosynthesis identified in plants (Cernac and Benning, 2004). However, the only lipid biosynthesis defect in the *wri1* alleles was a severe depletion in storage lipids of maturing seeds (Focks and Benning, 1998). Seed germination and seedling establishment were also altered in these mutants (Cernac et al.,

Figure 4. (continued).

(B) Accumulation of *WRI* mRNAs in mutant *wri* seeds was quantified 12 DAA by quantitative RT-PCR and presented as percentage of the constitutive *EF1α4* gene expression. Values are the means and SE of three replicates performed on three independent cDNA preparations obtained from batches of seeds dissected from four to five siliques. The three silique sets were harvested on distinct individuals.

(C) The fatty acid concentrations of dry seeds obtained from *wri* mutants were determined by gas chromatography analysis. Values are the means and SE of five replicates performed on batches of 20 seeds from five distinct individuals. DW, dry weight.

(D) The expression levels of two fatty acid biosynthetic genes, *BCCP2* and *PDH-E1a*, were investigated in developing seeds aged 12 DAA in different *wri* mutant lines by quantitative RT-PCR and presented as percentage of the constitutive *EF1α4* gene expression. Values are the means and SE of three replicates performed on cDNA dilutions obtained from three independent mRNA extractions.

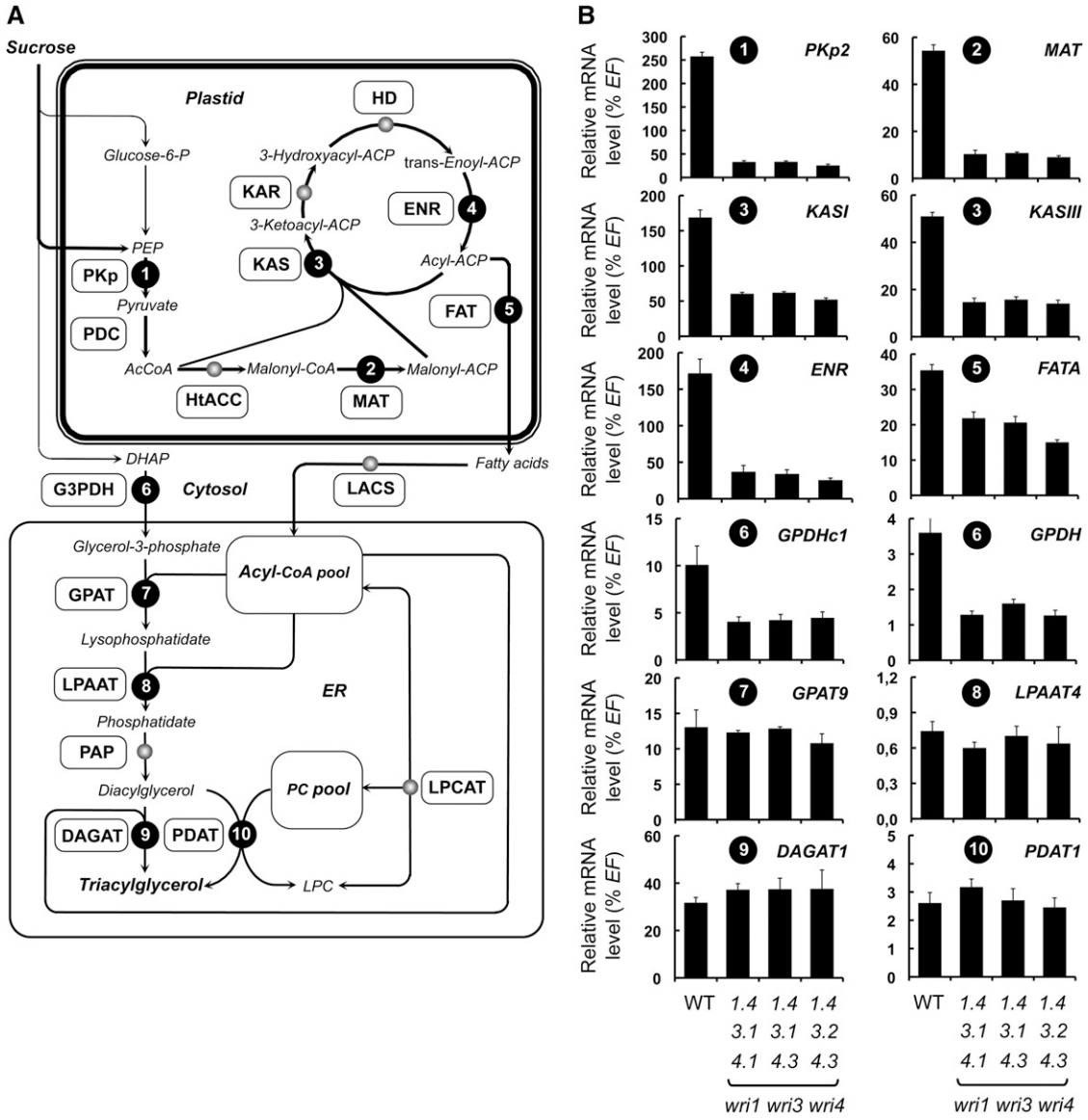

Figure 5. Expression of Genes Involved in Fatty Acid Biosynthesis and TAG Assembly in Maturing Seeds of *wri1 wri3 wri4* Mutants.

(A) Simplified scheme of carbon metabolism leading to TAG synthesis in maturing embryos of *Arabidopsis*. AcCoA, acetyl-CoA; ACP, acyl carrier protein; DAGAT, diacylglycerol acyltransferase; DHAP, dihydroxyacetone-3-phosphate; ENR, enoyl-ACP reductase; FAT, fatty acyl-ACP thioesterase; G3PDH, glycerol-3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; HD, 3-hydroxyacyl-ACP dehydratase; HtACC, heteromeric acetyl-CoA carboxylase; KAR, 3-ketoacyl-ACP reductase; KAS, 3-ketoacyl-ACP synthase; LACS, long-chain acyl-CoA synthetase; LPAAT, lysophosphaditic acid acyltransferase; LPC, lysophosphatidylcholine; LPCAT, acyl-CoA:lysophosphatidylcholine acyltransferase; MAT, malonyl-CoA:ACP transacylase; PAP, phosphatic acid phosphohydrolase; PC, phosphatidylcholine; PDAT, phospholipid:diacylglycerol acyltransferase; PDC, pyruvate dehydrogenase complex; PEP, phosphoenolpyruvate; PKp, plastidial pyruvate kinase.

(B) The expression patterns of genes encoding several enzymes of fatty acid or TAG biosynthetic pathways were investigated by quantitative RT-PCR and presented as percentage of the constitutive *EF1α4* (*EF*) gene expression. Values are the means and SE of three replicates performed on three independent cDNA preparations obtained from batches of seeds dissected from four to five siliques. The three silique sets were harvested on distinct individuals. WT, the wild type (Col-0).

2006), while further vegetative development and flowering were unmodified, suggesting that acyl chain production was not significantly altered during the majority of the plant life cycle. Accordingly, transcriptional activity of fatty acid biosynthetic genes was not compromised in the vegetative tissues of *wri1* plants. These observations led to the conclusion that the transcriptional machinery regulating the fatty acid biosynthetic pathway may contain distinct and partially redundant factors. The yeast one-hybrid screening procedure performed in this study allowed the identification of two new transcription factors regulating the fatty

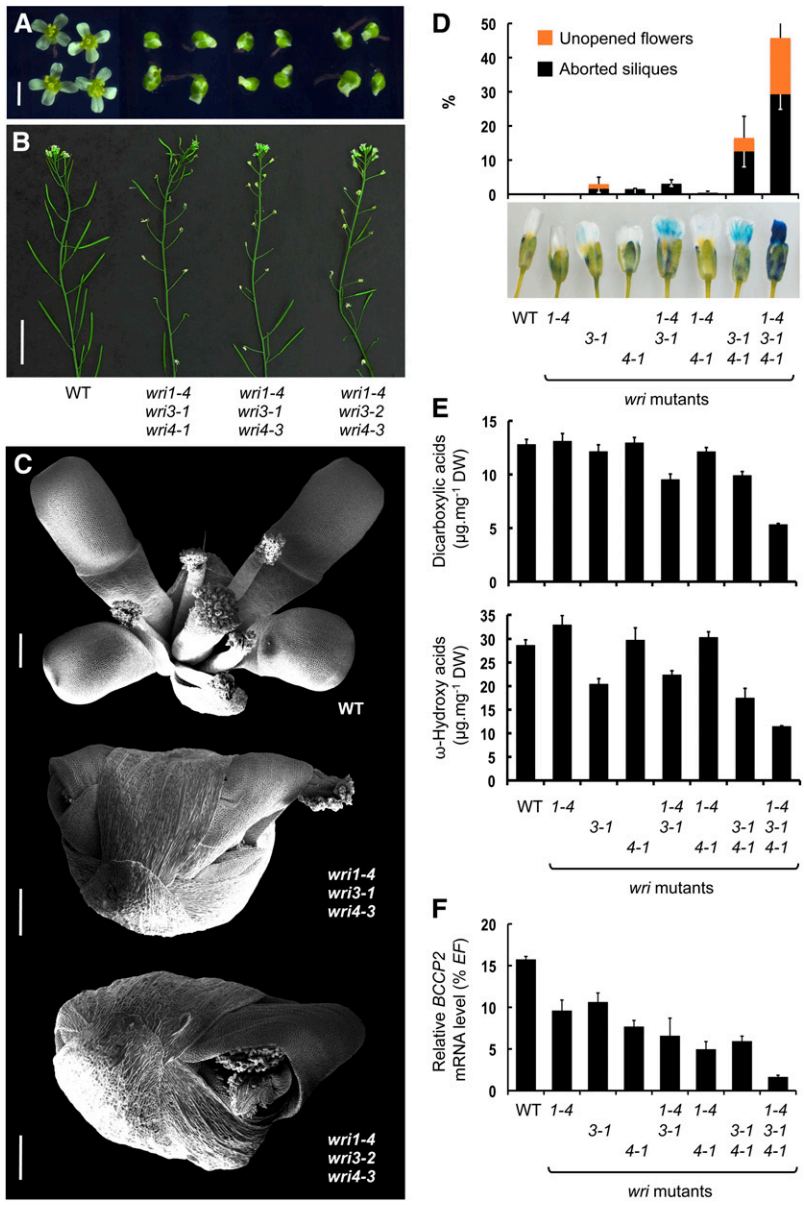

Figure 6. Flower Phenotypes of *wri1 wri3 wri4* Mutants Resulting from Altered Cutin Synthesis in Petals and Sepals.

(A) Microscopy observations of wild-type and mutant flowers. Bar = 2 mm.
(B) Observations of wild-type (WT) and mutant inflorescence stems. Bar = 2 cm.
(C) Scanning electron micrographs of wild-type and mutant flowers. Bars = 300 µm.
(D) Flower phenotypes and floral tissue permeability. Percentages of unopened flowers and aborted siliques on inflorescence stems of wild-type and mutant lines are presented. Values are the means and SE of observations performed on five different plants. On average, 100 to 150 flowers were observed for each plant considered. Permeability to toluidine blue of the epidermis of petals and sepals of wild-type and mutant lines is presented (bottom panel). Representative flowers are shown.
(E) Cutin monomer composition of flowers in a set of *wri* mutant lines: sum of all dicarboxylic and ω-hydroxy acids calculated on a dry weight basis of delipidated material. Values are the means and SE of three replicates performed on different plants.
(F) Accumulation of *BCCP2* mRNA in sepals and petals measured by quantitative RT-PCR. The results obtained were standardized to the constitutive *EF1αA4* (*EF*) gene expression level. Values are the means and SE of three to six replicates performed on cDNA dilutions obtained from three independent mRNA extractions.

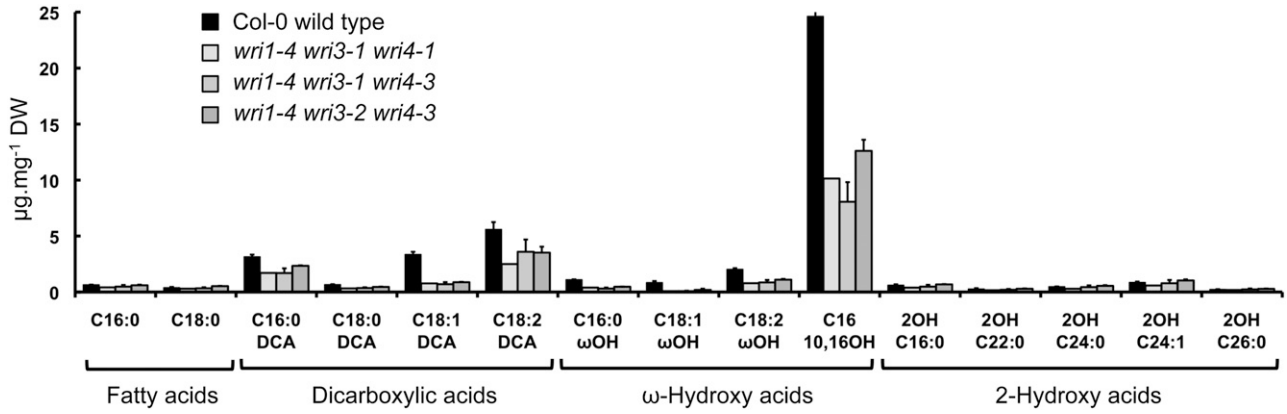

Figure 7. Cutin Production in the Flowers of *wri1 wri3 wri4* Mutants.

Amounts of cutin monomers are expressed on a dry weight basis of delipidated material. Each constituent is designated by carbon chain length and labeled by chemical class along the *x* axis. Values are the means and SE of three replicates performed on different plants. DW, dry weight.

acid biosynthetic pathway, WRI3 and WRI4. They belong to the AP2-EREBP family of transcription factors, and they are closely related to WRI1. Like WRI1, they are able to interact with promoter sequences of *BCCP1* and *BCCP2* in yeast, while their ectopic expression in transgenic *Arabidopsis* leads to the overaccumulation of several fatty acid biosynthetic mRNAs. Complementation of the *wri1-4* phenotype by seed-specific expression of either *WRI3* or *WRI4* ultimately demonstrated the ability of both factors to efficiently activate the whole fatty acid biosynthetic pathway. Whereas Cernac and Benning (2004) have reported that expression of *WRI1* cDNA under the control of the cauliflower mosaic virus 35S promoter leads to slightly increased seed oil content, neither the expression of *Pro35Sdual*:*WRI1* and *ProS2*:*WRI1* transgenes in a wild-type background (Baud et al., 2009) nor the introduction of *ProAT2S2*:*WRI1* in a *wri1-4* mutant background (this study) leads to efficient over accumulation of oil in the corresponding transgenic seeds. Several factors like growth conditions or strength and specificity of the different promoters used may explain these apparent discrepancies.

The high sequence similarity observed between the AP2 DNA binding domains of WRI1, WRI3, and WRI4 proteins suggests that the three factors may recognize and bind to a similar *cis*-regulatory element previously identified (Maeo et al., 2009). If the related WRI2 protein also possesses transcriptional activity (Figure 2A), this factor is neither able to interact with the promoter sequences of the fatty acid biosynthetic genes tested (Figures 1B and 1C) nor able to activate the corresponding biosynthetic pathway (Figures 2B to 2D). One explanation for this may reside in the amino acid sequence of the second AP2 domain of WRI2 which, in contrast with the first AP2 domain, strongly diverges from the other WRIs and may consequently impair the binding of WRI2 to the *cis*-elements recognized by WRI1, WRI3, and WRI4 (see Supplemental Figure 7 online). Nevertheless, *WRI2* mRNA was detected in many plant tissues (see Supplemental Figure 8A online), and a strong induction of this gene was observed in drying siliques. If WRI2 cannot activate fatty acid biosynthesis, the question of its ability to disrupt a functional transcription factor complex involving other WRIs remains an open question. Many transcriptional activators achieve their function by acting as part of multiprotein complexes, and truncated or modified versions of these transcription factors have been reported to prevent the formation of activating complexes and/or to interfere with subsequent transcriptional activation (Kim et al., 2008; Wang et al., 2009; Zhang et al., 2009). That said, the fine characterization of *wri2* T-DNA insertion alleles and of *WRI2* overexpressing lines allows us to rule out any negative effect of WRI2 on transcriptional activation of fatty acid biosynthesis and oil accumulation in seeds (see Supplemental Figures 8B to 8E online). Therefore, it is tempting to speculate that the function of WRI2, if any, may not be related to fatty acid biosynthesis, and further studies are required to elucidate the role of this transcription factor.

WRI Transcription Factors Are Essential to Ensure Proper Floral Organ Formation

The fine characterization of the collection of *wri* mutants has allowed us to establish a new physiological consequence of deficiency in *WRI1*-like genes. In developing petals and sepals, WRI1, WRI3, and WRI4 are implicated in the activation of the fatty acid biosynthetic pathway. High rates of acyl chain production in these tissues appear to be essential for cutin biosynthesis. The lack of the WRI transcription factors led to significant decreases in the amount of major cutin monomers produced by *wri1 wri3 wri4* flowers. Indeed, the main cutin monomer in flowers (10,16-dihydroxypalmitate) was decreased by more than 50%. Cutin is an insoluble polymer composed of interesterified fatty acids. Present at the surface of epidermal cells, cutin is embedded and overlaid with waxes and constitutes the structural component of the cuticle. In petals and sepals, this thick extracellular lipid barrier not only increases protection against dehydration, but also prevents the adhesion of floral organs between each other during flower development. In *wri1 wri3 wri4* flowers, reduced cutin production altered tissue permeability and dramatically increased the susceptibility of flowers to organ fusion, leading to semisterility. Similar

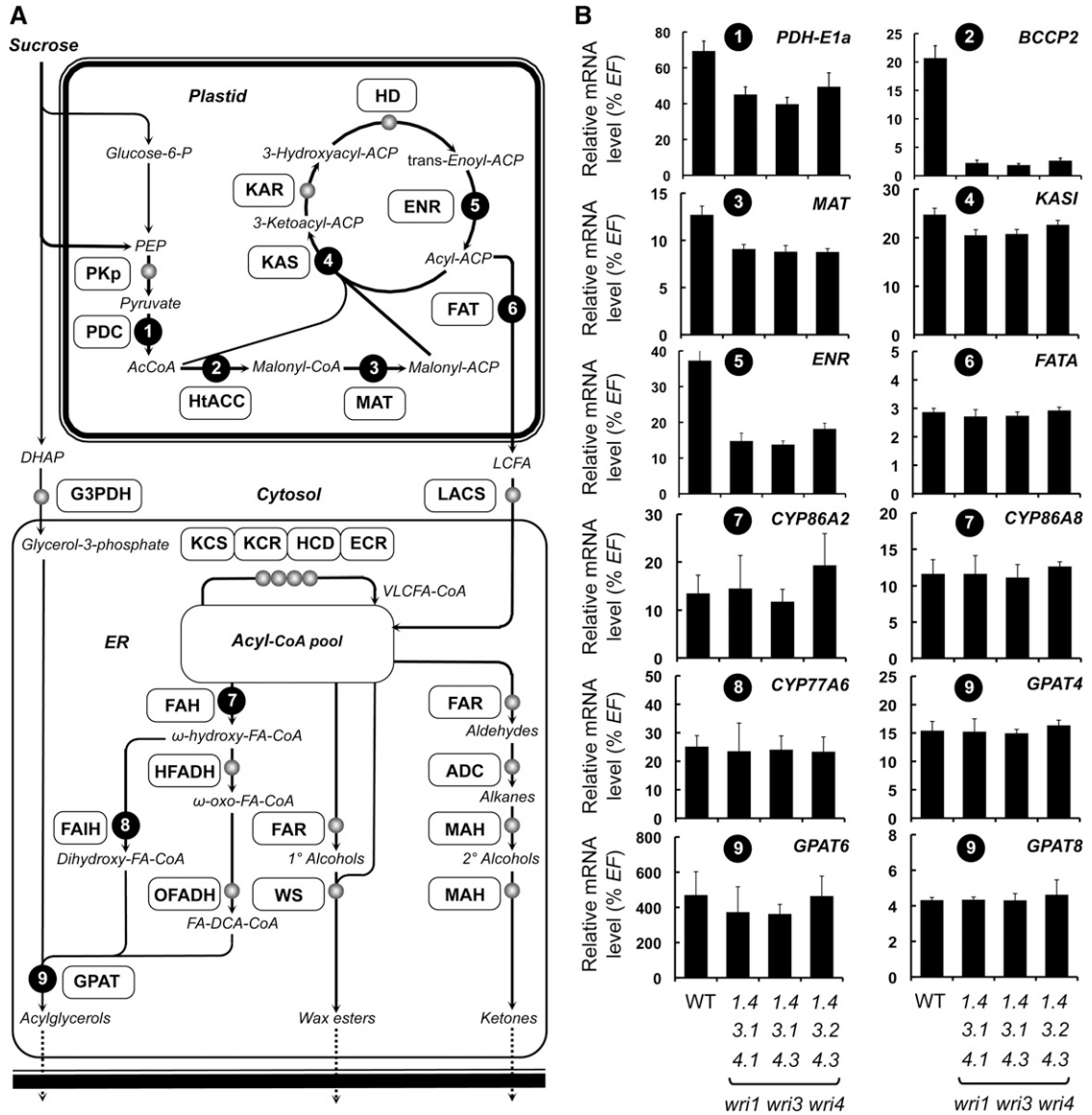

Figure 8. Expression of Genes Involved in Fatty Acid Biosynthesis and Cuticle Production in Petals and Sepals of *wri1 wri3 wri4* Mutants.

(A) Simplified scheme of carbon metabolism leading to cuticle synthesis in sepals and petals of *Arabidopsis*. AcCoA, acetyl-CoA; ACP, acyl carrier protein; ADC, aldehyde decarbonylase; DCA, dicarboxylic acid; ECR, enoyl-CoA reductase; ENR, enoyl-ACP reductase; FA, fatty acid; FAH, fatty acid hydroxylase; FAIH, fatty acyl in-chain hydroxylase; FAR, fatty acid reductase; FAT, fatty acyl-ACP thioesterase; G3PDH, glycerol-3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; HCD, hydroxyacyl-CoA dehydrogenase; HD, 3-hydroxyacyl-ACP dehydratase; HFADH, ω-hydroxy fatty acyl dehydrogenase; HtACC, heteromeric acetyl-CoA carboxylase; KAR, 3-ketoacyl-ACP reductase; KAS, 3-ketoacyl-ACP synthase; KCR, ketoacyl-CoA reductase ; KCS, ketoacyl-CoA synthase; LACS, long-chain acyl-CoA synthetase; LCFA, long-chain fatty acid; MAH, mid-chain alkane hydroxylase; MAT, malonyl-CoA:ACP transacylase; OFADH, ω-oxo fatty acyl dehydrogenase; PDC, pyruvate dehydrogenase complex; PEP, phosphoenolpyruvate; PKp, plastidial pyruvate kinase; VLCFA, very-long-chain fatty acid; WS, wax synthase.

(B) The expression patterns of genes encoding several enzymes of fatty acid or cuticle biosynthetic pathways were investigated by quantitative RT-PCR and presented as percentage of the constitutive *EF1α4* (*EF*) gene expression. Values are the means and SE of three replicates performed on cDNA dilutions obtained from three independent mRNA extractions. WT, the wild type (Col-0).

phenotypes have been reported for mutants affected in genes encoding enzymes involved in cuticle and wax production or proteins participating in the transport of these molecules (Wellesen et al., 2001; Panikashvili et al., 2010). Interestingly, another group of three transcription factors of the AP2-EREBP family (namely, SHINE1/WAX INDUCER1, SHINE2, and SHINE3) was previously shown to regulate cutin biosynthesis and to ensure proper floral organ morphology and surface formation (Broun et al., 2004; Shi et al., 2011). Whereas the WRI transcription factors activate genes involved in de novo fatty acid production

in the plastids, SHINE targets encode proteins located further downstream in the cutin biosynthetic network (e.g., formation of acyl-CoA species in the cytosol or fatty acid elongation within the endoplasmic reticulum) (Kannangara et al., 2007). However, members of the SHINE family not only regulate genes encoding cutin biosynthetic enzymes, they also control epidermal cell wall formation through regulating pectin metabolism and structural proteins (Kannangara et al., 2007; Shi et al., 2011). Another transcription factor, MYB30, has been proposed to regulate the biosynthesis of very-long-chain fatty acids and very-long-chain fatty acid–derived lipids, such as cuticular components during the incompatible interaction between *Arabidopsis* and avirulent bacterial pathogens (Raffaele et al., 2008). The list of the putative targets of MYB30, which comprises fatty acid–modifying enzymes and lipid assembling enzymes, is not redundant with that of the WRI family. To date, WRIs thus remain the only known transcriptional regulators of de novo fatty acid synthesis in plants.

WRI1 Is the Only Member of the WRI Clade Triggering High Rates of Acyl Chain Production in Seeds

The involvement of the three WRIs in fatty acid metabolism of petals and sepals is consistent with the comparable *WRI* mRNA levels measured in these organs. However, a characterization of the expression patterns of the *WRI* genes revealed contrasted profiles within some tissues. In developing seeds, for instance, *WRI1* was dramatically induced, whereas *WRI3* and *WRI4* mRNAs were barely detectable (Lee et al., 2009; this study). In agreement with these observations, WRI1 was the only member of the family participating in the activation of fatty acid biosynthesis in maturing embryos to provide acyl chains for TAG production. These observations show that the respective importance of the WRIs in a given tissue is proportional to their relative mRNA accumulation. The functional dissection and comparison of the three *WRI* promoters would be essential to understand both the basal activity of the *WRI*s in some plant organs and their specific induction in other tissues. Of particular interest would be the elucidation of the activation of *WRI1* in seeds. Previous reports have shown that master regulators of the maturation process like LEAFY COTYLEDON1 (LEC1) or LEC2 could directly regulate *WRI1* transcription (reviewed in Baud and Lepiniec, 2010). However, a close examination of the *WRI1* minimal promoter reveals that this nucleotide sequence lacks the *cis*-regulatory elements usually recognized by these transcription factors. Further studies are under way to elucidate this original and as yet unknown transcriptional regulatory process involving some of the key master regulators of seed maturation.

Missing Elements in Our Current Understanding of the Regulation of Fatty Acid Biosynthesis in Plants

In maturing embryos, as in floral organs, WRI targets consist of enzymes catalyzing late glycolysis and de novo fatty acid synthesis, whereas most enzymes involved in fatty acid modification or lipid (TAG in the seeds or cutin in floral organs) assembly escape this regulation. Importantly, two genes encoding glycerol-3-phosphate dehydrogenase, the enzyme providing glycerol backbones for the synthesis of TAGs, are also downregulated in the absence of the WRIs (Figure 5). These results are consistent with recent transcriptomic data obtained with transgenic maize lines overexpressing Zm *Wri1a*, one of the orthologs of At *WRI1* in maize (Pouvreau et al., 2011). Members of the WRI family thus behave as transcriptional enhancers differentially activated during the course of plant development to provide precursor molecules (acyl chains and glycerol backbones) for lipid biosynthesis. The expression of genes involved in lipid assembling pathways is activated by distinct transcription factors, including members of the SHINE family, MYB30, or master regulators of the seed maturation program (e.g., LEC2). The downregulation of *ROD1* observed in the triple mutants highlights the importance of now identifying direct targets of the WRIs in an exhaustive manner, by means of chromatin immunoprecipitation strategies for instance. If the analysis of *Arabidopsis wri* mutants has allowed us to show the importance of the WRIs in the production of seed TAGs or floral cutin, the expression patterns of the WRIs suggest that they might be involved in the production of different types of lipids in other tissues (e.g., pollen grains or nectaries). The limited size of our model species seriously hampers lipidomic analyses of such tissues, but other plant systems could be used for further investigations (e.g., rapeseed).

If the fine characterization of the triple *wri1 wri3 wri4* mutants revealed severe defects in flower development and seed filling, it is interesting to note that transcription of fatty acid biosynthetic genes was not completely abolished in these mutant backgrounds. Accordingly, and once seedling establishment was achieved, vegetative growth of the mutant plants was not altered, at least under controlled growth conditions. These observations demonstrate that the WRIs are transcriptional enhancers of the fatty acid biosynthetic pathway triggering high rates of acyl chain production when/where needed, while other actors may ensure basal transcriptional activity of the fatty acid biosynthetic genes. Both the identity and the mode of action of these actors remain unknown. So far, it is not possible to exclude that other AP2/EREPB proteins related to the WRIs could substitute for them. However, the occurrence of *cis*-regulatory elements different from the AW-box bound by WRI1 (Maeo et al., 2009) and essential for the activation of fatty acid biosynthetic genes (Baud et al., 2009) suggests the involvement of other classes of transcriptional regulators.

Finally, alterations (resulting from T-DNA insertions or overexpression strategies) of *WRI1* (Cernac et al., 2006) or *WRI3* (Lee et al., 2009) expression levels in *Arabidopsis* have been shown to interfere with abscisic acid signaling, especially in germinating seedlings. Complementary studies would be required to elucidate the molecular mechanism(s) linking fatty acid production and abscisic acid–related phenotypes.

Conclusion and Future Prospects

In summary, we show here that WRI1, WRI3, and WRI4 define a class of AP2-EREBP transcription factors involved in the transcriptional activation of the fatty acid biosynthetic pathway in *Arabidopsis*. Their clearly different expression patterns allow triggering of high rates of acyl chain production in a developmentally

regulated manner, thus providing precursors for various lipid biosynthetic pathways. In particular, the demonstration of cutin-deficient phenotype for the triple mutants establishes a new physiological consequence of deficiency in *WRI1*-like genes. This study then suggests that the WRIs behave as transcriptional enhancers, and further work will be required to isolate and characterize transcriptional regulators responsible for basal activation of the fatty acid biosynthetic pathway, if any. Ultimately, the full elucidation of the regulation of fatty acid production in plants will require the further deciphering of posttranscriptional regulations fine-tuning this biosynthetic network. For instance, recent studies have established that plastidial heteromeric acetyl-CoA carboxylase, considered as a key regulatory enzyme of the fatty acid biosynthetic pathway, is subjected to a negative regulation mediated either by regulatory proteins (PII; Feria Bourrellier et al., 2010) or by products of the fatty acid synthase complex (18:1-ACP; Andre et al., 2012).

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana seeds of Col-0 and Wassilewskija accessions were obtained from the *Arabidopsis thaliana* resource center for genomics at the Institut Jean-Pierre Bourgin, and T-DNA mutant lines were ordered from the Salk Institute. Plants were cultured as described previously (Baud et al., 2007a). Phenotyping of the mutants was performed in growth chambers (16-h-light photoperiod, 18°C night/21°C day, and 65% relative humidity). Material used for RNA extraction was frozen in liquid nitrogen immediately after harvest and then stored at −80°C prior to extraction.

Molecular Characterization of T-DNA Mutants

Plant genomic DNA flanking the left T-DNA borders of the Salk mutants were amplified by PCR and sequenced to confirm the FSTs identified. Molecular characterization of *wri1-3* and *wri1-4* alleles was previously described (Baud et al., 2007b). In the *wri3-1* line (N656326), the T-DNA insertion was located in the 3rd intron of the *WRI3* gene. To amplify the T-DNA left border, WL2R1 (5′-AGTAGCAAAAATGTTTGGGG-3′) and SigLB2 (5′-TGTTGCCCGTCTCACTGG-3′) were used. The right T-DNA border could not be attained in this line. To amplify the T-DNA left border in *wri3-2* (N625491; insertion located in the 7th intron of the *WRI3* gene), WL2R3 (5′-GTTTTACTCCCCAAACTATTATG-3′) and SigLB2 were used. The opposite border was amplified with WL2F3 (5′-GCATTTGAGTTTATTAGTGGTTAG-3′) and SigLB1 (5′-CGGAACCACCATCAAACAG-3′); this border appeared to be a second left border, indicating that a complex insertion event had occurred in the *wri3-2* line. In the *wri4-1* line (N518113), the T-DNA insertion was located in the 2nd exon of the *WRI4* gene. To amplify the T-DNA left border, WL3R1 (5′-CTCACCTAGATAAACTGCACTTG-3′) and SigLB2 were used. The right T-DNA border could not be attained. To amplify the T-DNA left border in *wri4-2* (N505384; insertion located in the 7th intron of the *WRI4* gene), WL3F3 (5′-ATAATGGGAGATGGGAAG-3′) and SigLB2 were used. The opposite border was amplified with SigLB1 and WL3R3 (5′-GTTCGTTTGACTACATTCATG-3′); this border appeared to be a second left border, indicating that a complex insertion event had occurred in the *wri4-2* line. In the *wri4-3* line (N546920), the T-DNA insertion was located in the 8th exon of the *WRI4* gene. To amplify the T-DNA left border, WL3F5 (5′-GATCAGATGACATGCATATTTC-3′) and SigLB2 were used. The right T-DNA border could not be attained in this line. In the *wri2-1* line (N838658), the T-DNA insertion took place in the 2nd exon of the *WRI2* gene. To amplify the T-DNA left border, WL1F0 (5′-GGAGCTAAATTATCTGGAATCTG-3′) and SAILLB1 (5′-GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC-3′) were used. The right T-DNA border could not be attained in this line. To amplify the T-DNA left border in *wri2-2* (N611105; insertion located in the 6th exon of the *WRI2* gene), WL1R2 (5′-GTATGAATGTTAATTGTGAGAAAG-3′) and SigLB1 were used. The opposite border was amplified with SigLB1 and WL1F2 (5′-CACTGTTTTTAGCTACATGC-3′); this border appeared to be a second left border, indicating that a complex insertion event had occurred in the *wri2-2* line.

Constructs and Plant Transformation

Construction of the ProWRI:uidA Transgenes

For construction of the *ProWRI3*:*uidA* transgene, region −1000 to −1 bp relative to the *WRI3* translational start codon was amplified with the proofreading Pfu Ultra DNA polymerase (Stratagene) from Ws genomic DNA using 5′-*att*B1-TCCATCTAGTTAAGGGGCAG-3′ and 5′-*att*B2-CAAAAGAAGAGGAAGAAGAAGA-3′, *att*B1 and *att*B2 referring to the corresponding Gateway recombination sequences. The PCR product was introduced by BP recombination into the pDONR207 entry vector (Invitrogen) and transferred into the destination vector pBI101-R1R2-GUS (Baud et al., 2007b) by LR recombination.

For construction of the *ProWRI4*:*uidA* transgene, a similar procedure was adopted. Primer sequences were as follows: 5′-*att*B1-TCTTAAATGCTTACTTATGATTTCAG-3′ and 5′-*att*B2-CTACCGGTGAATGAGAAGA-3′. Construction of the *ProWRI1*:*uidA* transgene was previously described (Baud et al., 2009). Resulting binary vectors were electroporated into *Agrobacterium tumefaciens* C58C^1 strain and used for agroinfiltration of flower buds of *Arabidopsis* (Bechtold et al., 1993). Primary transformants were selected on Murashige and Skoog (MS) medium containing kanamycin (50 mg·L^{-1}) and transferred to soil for further characterization. For each construct, between 17 and 19 independent transgenic lines were considered.

Construction of the Pro35Sdual:WRI Transgenes

For construction of the *Pro35Sdual*:*WRI2* transgene, *WRI2* cDNA was amplified with 5′-*att*B1-ATGGCGTCGGTGTC-3′ and 5′-*att*B2-TCATTTCTCTTGTGGGAGG-3′, cloned in pDONR207, and finally transferred to the binary vector pMDC32 (Curtis and Grossniklaus, 2003) as previously described. For construction of *Pro35Sdual*:*WRI3* and *Pro35Sdual*:*WRI4* transgenes, a similar procedure was adopted. Primer sequences were as follows: 5′-*att*B1-TCATGTTCATCGCCGTCGAA-3′ (*WRI3* atg) and 5′-*att*B2-CTTAGCAATCATTTAACTCGC-3′ (*WRI3* stop), 5′-*att*B1-TCATGGCAAAAGTCTCTGG-3′ (*WRI4* atg) and 5′-*att*B2-CTTAAGGTCCATAATCAAACTC-3′ (*WRI4* stop). Construction of the *Pro35Sdual*:*WRI1* transgene was previously described (Baud et al., 2007b). Resulting binary vectors were electroporated into *Agrobacterium* C58C^1 strain and used for agroinfiltration of *wri1-4* plants. Primary transformants were selected on MS medium containing hygromycin (50 mg·L^{-1}) and transferred to soil for further characterization.

Construction of ProAT2S2:WRI Transgenes

WRI cDNAs were transferred from pDONR207 to the binary vector ProAT2S2-R1R2-HYGRO (Pouvreau et al., 2011) by LR recombination. The corresponding binary vectors were electroporated into *Agrobacterium* C58C^1 strain and used for agroinfiltration of *wri1-4* flower buds. Primary transformants were selected on MS medium containing hygromycin (50 mg·L^{-1}). The progeny of these primary transformants (T2 seeds) was subjected to segregation analyses for hygromycin resistance, and lines segregating 3:1 were selected (heterozygous lines, one insertion locus). T2 lines were then grown in a greenhouse, and their progeny (T3

seeds) were subjected to segregation analyses. Lines producing 100% resistant plantlets were selected (homozygous lines, single insertion locus) and used for further characterization.

RNA Analyses

RNA extraction, reverse transcription, RT-PCR, and real-time quantitative RT-PCR were performed as previously described (Baud et al., 2004). Primers used for RT-PCR (Figure 4A) were as follows: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAAGAAGCGCTTAACCACT-3′ (*WRI1* atg) and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGACCAAATAGTTACAAGA-3′ (*WRI1* stop), *WRI3* atg and *WRI3* stop (see above), *WRI4* atg and *WRI4* stop (see above), 5′-ATGCCCCAGGACATCGTGATTTCAT-3′ (*EF* up) and 5′-TTGGCGGCACCCTTAGCTGGATCA-3′ (*EF* low). The sequences of primers used for real-time quantitative RT-PCR are indicated in Supplemental Table 1 online.

Yeast One-Hybrid Experiments

The pHISi reporter plasmid containing a 180-bp fragment of the *BCCP2* promoter was constructed as previously described (Baud et al., 2009). This plasmid was integrated into the yeast strains EGY48 (Estojak et al., 1995; for screening of the REGIA cDNA expression library) and YM4271 (Liu et al., 1993; for validation of the candidates). To screen the REGIA cDNA expression library, 8 mL of liquid yeast peptone dextrose adenine medium inoculated with a single prey colony was incubated overnight at 28°C with regular shaking. The yeast strains constituting the REGIA expression library (Paz-Ares, 2002) were grown on solid yeast peptone dextrose adenine and inoculated with 5 μL of prey culture. Mating of yeast strains was allowed by incubation at 28°C during 24 h. Successful mating cells were selected on synthetic dropout medium (SD)-Trp-Ura and then resuspended in 100 μL of sterile water. Finally, 5 μL of yeast suspension were transferred on screening plates (SD-Trp-Ura-His); several concentrations of 3-aminotriazole were tested, from 0 to 5 mM.

To validate the candidate genes isolated, *WRI1*, *WRI2*, *WRI3*, and *WRI4* cDNAs were cloned into the pDEST22 vector (Invitrogen) to be expressed in yeast as GAL4 activating domain fusions. cDNAs cloned into the pDONR207 (see above) were transferred into the pDEST22 expression vector by LR recombination. YM4271 yeast cells presenting the *HIS3* reporter gene under the control of a functional *BCCP2* promoter (see above) were transformed with pDEST22 according to the AcLi/SSDNA/PEG method (Gietz and Woods, 2002). Transformants were selected on appropriate media. Construction of *ProBAN:HIS* and *ProBCCP1:HIS* was described previously (Baud et al., 2010).

The reporter plasmid used for β-galactosidase assays was made by inserting a 180-bp fragment of the *BCCP2* promoter into the pLacZi vector (Luo et al., 1996). This fragment was amplified by PCR with the proofreading Pfu Ultra DNA polymerase (Stratagene) using 5′-GGAATTCCACAAAAGGAGCGGTTTGG-3′ and 5′-TCCGTCGACGGATGTTGAGACAGTGGACGATG-3′, digested by *Eco*RI and *Sal*I, and inserted into pLacZi between the *Eco*RI and *Sal*I sites. This plasmid was then digested with *Apa*I and integrated into the yeast strain YM4271 at the *URA3* locus. The resulting yeast strains, selected on a medium lacking uracil, were then cotransformed with the pDEST22 vector allowing expression of WRI1, WRI2, WRI3, or WRI4 using the AcLi/SSDNA/PEG method (Gietz and Woods, 2002). Transformants were selected on appropriate media and β-galactosidase activity was measured on liquid cultures using *o*-nitrophenyl β-D-galactopyranoside as a substrate, as recommended by the constructor (Yeast Protocol Handbook; Clontech).

To determine whether the candidate transcription factors isolated possessed transcriptional activity, *WRI1*, *WRI2*, *WRI3*, and *WRI4* cDNAs were cloned into the pDEST32 vector (Invitrogen) to be expressed in yeast as fusions with GAL4-DBD. The cDNAs previously cloned in pDONR207 (see above) were transferred into the pDEST32 expression vector by LR recombination. AH109 yeast cells were transformed with pDEST32 according to the AcLi/SSDNA/PEG method (Gietz and Woods, 2002). Transformants were selected on appropriate media.

Lipid Analyses

For determination of seed oil content, total fatty acid analyses were performed on pools of 20 dry seeds as previously described (Li et al., 2006). For determination of flower cutin polyester composition, tissues were collected and immersed in hot isopropanol for 10 min at 80°C. After cooling, samples were extensively delipidated by extracting the soluble lipids, then dried and depolymerized. Extraction, derivatization, and analysis were performed as previously described (Domergue et al., 2010).

Microscopy

To obtain scanning electron micrographs, dry seeds or freshly harvested flowers were mounted onto a Peltier cooling stage using adhesive discs (Deben) and observed with a SH-1500 tabletop scanning electron microscope (Hirox). Histochemical detection of GUS activity and bright-field microscope observations were performed as described by Baud et al. (2007a). To test the permeability of floral tissues, flowers without stamens and pistils were dipped in a 0.05% (w/v) toluidine blue solution containing 0.01% (w/v) Tween 20 for 30 min, rinsed twice in distilled water prior to bright-field microscopy observations (Li-Beisson et al., 2009).

Phylogenetic Analysis

To perform distance analysis among AP2 transcription factors of the AP, AINTEGUMENTA, and WRI families, programs (with default values) proposed at http://mafft.cbrc.jp/alignment/server/ were used: MAFFT (Multiple Alignment using Fast Fourier Transform) version 6 for multiple alignments and NJ (Neighbor Joining, with 1000 bootstraps) for tree building. Alignments are provided in Supplemental Data Set 1 online, and a version of the distance tree presenting statistical support for nodes is provided as Supplemental Figure 9 online.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *At2S2*, At4g27150; *BCCP1*, At5g16390; *BCCP2*, At5g15530; *CYP77A6*, At3g10570; *CYP86A2*, At4g00360; *CYP86A8/LCR*, At2g45970; *DAGAT1*, At2g19450; *ENR*, At2g05990; *EF1αA4*, At5g60390; *FAE1/KCS18*, At4g34520; *FAD2*, At3g12120; *FAD3*, At2g29980, *FATA*, At3g25110; *GPAT*, At5g60620; *GPAT4*, At1g01610; *GPAT6*, At2g38110; *GPAT8*, At4g00400; *GPDH*, At3g07690; *GPDHc1*, At2g41540; *KASI*, At5g46290; *KASIII*, At1g62640; *LPAAT4*, At1g75020; *MAT*, At2g30200; *PDAT1*, At3g44830; *PDH-E1a*, At1g01090; *PKp2*, At5g52920; *ROD1*, At3g15820; *WRI1*, At3g54320; *WRI2*, At2g41710; *WRI3*, At1g16060; and *WRI4*, At1g79700.

Supplemental Data

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

Supplemental Figure 1. Relative Accumulation of *WRI* mRNAs in Siliques of Transgenic Lines.

Supplemental Figure 2. Expression of Genes Involved in Fatty Acid Modification in Maturing Seeds of *wri1 wri3 wri4* Mutants.

Supplemental Figure 3. Complementary Results for the Characterization of the Flower Phenotype of *wri* Mutants.

Supplemental Figure 4. Cutin Analyses of Inflorescence Stems and Rosette Leaves of the *wri1-4 wri3-1 wri4-1* Mutant.

Supplemental Figure 5. Cuticular Wax Composition of Wild-Type and *wri1 wri3 wri4* Flowers.

Supplemental Figure 6. *WRI* Relative mRNA Levels of *wri* Mutant Floral Organs.

Supplemental Figure 7. Alignment of Amino Acid Sequences Corresponding to the DNA Binding Domains of WRI1, WRI2, WRI3, and WRI4.

Supplemental Figure 8. Characterization of *WRI2*.

Supplemental Figure 9. Version of the Phylogram among AP2 Transcription Factors of the APETALA, AINTEGUMENTA, and WRINKLED Clades Presenting Statistical Support for Nodes.

Supplemental Table 1. Primers Used for Quantitative RT-PCR.

Supplemental Data Set 1. Text File of the Alignment of Amino Acid Sequences Corresponding to the Double AP2 Domains of the Transcription Factors of the APETALA, AINTEGUMENTA, and WRINKLED Clades.

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AUTHOR CONTRIBUTIONS

J.J. performed the research and analyzed the data. G.B., A.L., and A.S. performed the research. S.J. and L.L. designed the research and analyzed the data. A.T. and S.B. designed and performed the research, analyzed the data, and wrote the article.

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