TRANSPARENT TESTA8 Inhibits Seed Fatty Acid Accumulation by Targeting Several Seed Development Regulators in Arabidopsis[1][C][W]

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Fatty acids (FAs) and FA-derived complex lipids play important roles in plant growth and vegetative development and are a class of prominent metabolites stored in mature seeds. The factors and regulatory networks that control FA accumulation in plant seeds remain largely unknown. The role of *TRANSPARENT TESTA8* (*TT8*) in the regulation of flavonoid biosynthesis and the formation of seed coat color is extensively studied; however, its function in affecting seed FA biosynthesis is poorly understood. In this article, we show that Arabidopsis (*Arabidopsis thaliana*) *TT8* acts maternally to affect seed FA biosynthesis and inhibits seed FA accumulation by down-regulating a group of genes either critical to embryonic development or important in the FA biosynthesis pathway. Moreover, the *tt8* mutation resulted in reduced deposition of protein in seeds during maturation. Posttranslational activation of a TT8-GLUCOCORTICOID RECEPTOR fusion protein and chromatin immunoprecipitation assays demonstrated that TT8 represses the activities of *LEAFY COTYLEDON1*, *LEAFY COTYLEDON2*, and *FUSCA3*, the critical transcriptional factors important for seed development, as well as *CYTIDINEDIPHOSPHATE DIACYLGLYCEROL SYNTHASE2*, which mediates glycerolipid biosynthesis. These results help us to understand the entire function of *TT8* and increase our knowledge of the complicated networks regulating the formation of FA-derived complex lipids in plant seeds.

Fatty acids (FAs) and FA-derived complex lipids are the major energy reserve substance stored in the seeds of many higher plants. In Arabidopsis (*Arabidopsis thaliana*), the development of the seed requires the precise regulation of three distinct tissues: the embryo, endosperm, and seed coat. After the occurrence of double fertilization in the embryo sac, the formation of the embryo and endosperm from zygotic tissues is initiated. The accumulation of most seed FAs is physiologically coupled with seed development, which is generally divided into two processes: embryonic morphogenesis (EM) and maturation (Baud and Lepiniec, 2009). EM starts by double fertilization, undergoes a series of programmed cell division and differentiation, and ends at 6 d after pollination (DAP) with the formation of a torpedo-shaped embryo (Goldberg et al., 1994; Mayer and Jürgens, 1998; Jenik et al., 2007). The maturation phase takes place from 7 to 20 DAP and features the accumulation of seed storage reserves, such as seed lipids and proteins, and the acquisition of dormancy and desiccation tolerance (Goldberg et al., 1994; Baud et al., 2002; Fait et al., 2006). During the process of seed development, the endosperm supplies nutrition to the developing embryo. Finally, it is degraded and reduced to merely one cell layer surrounding the embryo, accounting for roughly 10% of the total seed FAs (Penfield et al., 2004; Li et al., 2006; Stone et al., 2008; Baud and Lepiniec, 2009). The seed FA accumulation that mainly occurs in embryonic tissues exhibits a sigmoid pattern, changing slightly at the beginning and end but drastically between 7 and 17 DAP.

The seed coat is derived from the maternal integument and consists of four cell layers, an endothelial layer, a crushed layer forming the inner integument, and an epidermis comprising the outer integument, from inside to outside (Debeaujon et al., 2000). The seed coat not only plays essential roles in nourishing the embryo and protecting it against adverse environmental conditions but also in determining the morphology, dormancy, and longevity of a seed (Mohamedyasseen et al., 1994; Weber et al., 1996; Debeaujon et al., 2000).

Flavonoids are one of the largest groups of secondary metabolites unique to higher plants, biosynthesized from malonyl-CoA and Phe. They can be classified into three major classes, flavonols, anthocyanins, and proanthocyanidins, which are modified from a 15-carbon skeleton. In plants, flavonoid derivatives determine the pigmentation pattern of vegetative organs and seeds (Nesi et al.,

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2001; Lepiniec et al., 2006) and are involved in multiple functions. For example, they play important roles in protecting plants against a wide range of environmental injuries (Debeaujon et al., 2000; Agati and Tattini, 2010; Agati et al., 2012; Chen et al., 2012b). Seed coat mutants include two major groups. One group, represented by the *transparent testa* (*tt*) and *transparent testa glabra* (*ttg*) defects in testa flavonoid accumulation, has changes in seed coat color ranging from yellow to pale brown or grayish brown (Shirley et al., 1995; Debeaujon et al., 2000). The second group, represented by *glabra2* (*gl2*), features abnormal testa structures, such as lacking the formation of mucilage (Rerie et al., 1994; Masucci and Schiefelbein, 1996), an important secreta produced by outer seed coat cells and composed primarily of a heterogenous group of acidic polysaccharides that form a gel-like matrix with two key pectins, homogalacturonan and rhamnogalacturonan I (Western et al., 2000; Macquet et al., 2007a, 2007b). The seed FA and flavonoid biosynthesis depends on the precise coordination of the embryo, endosperm, and seed coat. Therefore, the mutants defective in the development of the seed coat could also affect embryonic FA biosynthesis. Recent studies demonstrate that the *tt2* mutation resulted in a significant increase of seed FA content (Chen et al., 2012b), and the *gl2* mutation produced more seed oil due to increased carbon allocation to the embryo in the absence of seed coat mucilage biosynthesis (Shi et al., 2012).

Previous studies revealed that *TT8*, which encodes a basic helix-loop-helix domain regulatory protein in the nucleus, is involved in anthocyanin and proanthocyanidin biosynthesis and regulates the expression *DIHYDROFLAVONOL 4-REDUCTASE* and *BANYULS*, two genes in the flavonoid biosynthesis pathway (Nesi et al., 2000; Baudry et al., 2006). Despite our knowledge regarding the function of *TT8* in flavonoid metabolism, the role of *TT8* in seed FA biosynthesis has not been reported. Thus, we designed and performed a series of genetic and molecular experiments to investigate whether *TT8* is also involved in FA biosynthesis and to understand the mechanism by which TT8 targets the downstream factors to regulate seed development and FA biosynthesis. Our results clearly show that TT8 inhibits seed FA accumulation by targeting several transcription factors (TFs) regulating seed development in Arabidopsis.

RESULTS

TT8 Changes FA Storage and FA Composition in Mature Seeds

Two transfer DNA insertion mutant lines of the *TT8* gene, *tt8-3* (Nesi et al., 2000) and *tt8-4* (SALK_063334), a newly named allele in this study, were provided by the Arabidopsis Biological Resource Center. The structure of the *TT8* gene and the positions of the transfer DNA insertions in the *tt8-3* and *tt8-4* mutants are shown in Supplemental Figure S1A. Genotyping by PCR (Supplemental Fig. S1B) indicated the presence of the homozygous *tt8-4* mutant, which completely lacks the transcript of the *TT8* gene, as detected by reverse transcription (RT)-PCR (Supplemental Fig. S1C). The *tt8-3* and *tt8-4* mutants produce yellow seeds due to the lack of flavonoid deposition in the seed coats. The *tt8* mutant seeds are slightly bigger than their respective

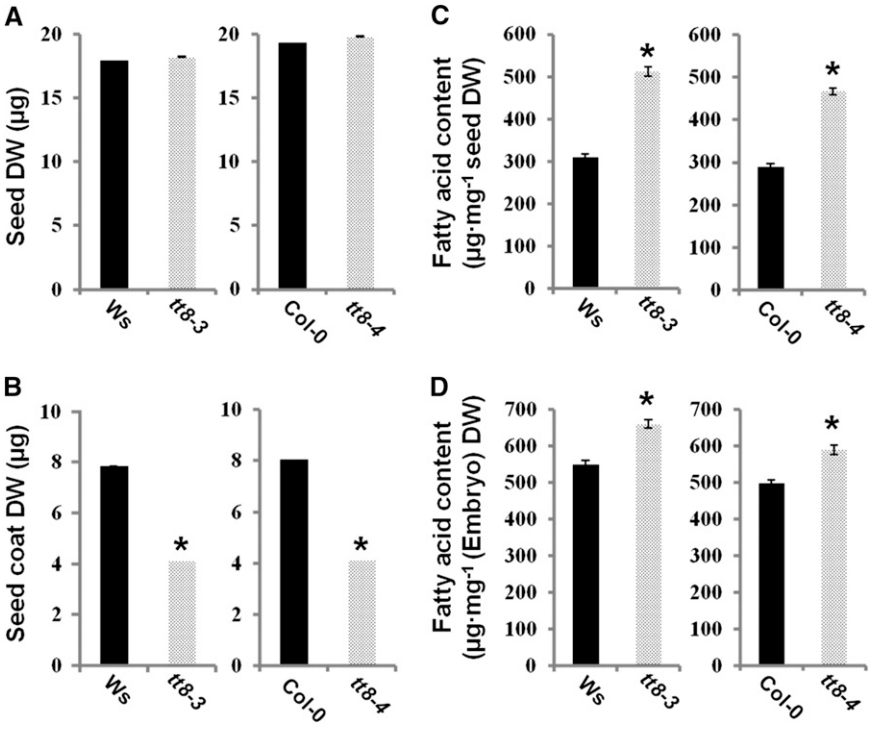

Figure 1. Comparison between the *tt8* mutant lines and their respective wild types. A, Seed dry weight (DW). B, Seed coat dry weight. C, FA content. D, FA content of the embryo. Data presented are means of three independent biological repeats ± SE. Asterisks indicate significant differences ($P \leq 0.05$) compared with the wild type.

Table I. *Comparison of FA composition and total FA content ($\mu g\ mg^{-1}$ seed dry weight) in mature seeds between tt8 mutant lines and their respective wild types*

Data reported are means of five independent experiments (five biological repeats) ± SE. Asterisks indicate significant differences ($P \leq 0.05$).

Line	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	24:0	Sum
Ws	29.4 ± 1.1	10.2 ± 0.9	27.0 ± 1.1	115.3 ± 2.3	83.1 ± 1.8	5.1 ± 0.1	39.9 ± 1.1	0.8 ± 0.1	3.7 ± 0.2	0.8 ± 0.1	315.1 ± 8.7
tt8-3	45.7 ± 1.6*	15.2 ± 0.6*	72.8 ± 1.3*	165.2 ± 2.7*	126.9 ± 2.3*	10.1 ± 0.1*	69.9 ± 1.2*	1.9 ± 0.8*	8.2 ± 0.2*	2.7 ± 0.1*	518.5 ± 10.9
Col-0	28.2 ± 0.9	8.5 ± 0.3	18.6 ± 0.5	109.5 ± 2.5	78.2 ± 1.3	3.9 ± 0.1	36.2 ± 0.6	0.8 ± 0.1	2.9 ± 0.8	0.5 ± 0.1	287.3 ± 7.2
tt8-4	43.8 ± 1.2*	12.1 ± 0.4*	65.9 ± 1.0*	153.7 ± 3.2*	117.2 ± 1.3*	7.4 ± 0.1*	61.1 ± 1.0*	1.6 ± 0.1*	6.6 ± 0.3*	1.5 ± 0.1*	471.0 ± 8.6

wild-type controls (Supplemental Fig. S2). However, no obvious differences in embryo size and cell size in the central region of the embryo between *tt8-4* and the wild type (Columbia-0 [Col-0]) were observed (Supplemental Fig. S3). The dry weight of *tt8* seeds upon maturation was also similar to that of the corresponding wild-type seeds (Fig. 1A). However, the dry weights of *tt8-3* and *tt8-4* embryos were 40.1% and 39.3% heavier than that of their respective wild-type controls, reflecting the differences in developmental effects. In contrast, the dry weight of seed coats of the *tt8* seeds was only 50% of that in the respective wild types (Fig. 1B).

The seed FA content and FA composition in mature *tt8* seeds were measured and are shown in Table I and Figure 1C. The FA content of *tt8* seeds was 60% higher than that of wild-type seeds. Regardless of which allele mutated, the increase in seed FA content was attributed to the elevated proportion of embryo weight as well as the promoted FA production in the embryos (Fig. 1, B and D). The increase of FA content was accompanied by an alteration in FA composition. In the mature *tt8* seeds, the proportions of 16-C and 18-C FAs, with an exception for 18:1, decreased significantly, whereas the proportions of very-long-chain FAs (C ≥ 20), including 20-C, 22-C, and 24-C FAs, increased significantly (Table II). The subcellular structures of the *tt8* mutant lines (*tt8-3* and *tt8-4*) were compared with those of Wassilewskija (Ws) and Col-0, respectively (Fig. 2). Figure 2 also indicates the average number of oil bodies that were contained in a cell and the mean lengths of long and short axes of an oil body. It is clear from the figure that there were smaller numbers and larger sizes of oil bodies in the *tt8* cells than in the wild-type cells. These results demonstrate that the *tt8* mutation results in defective flavonoid accumulation in the seed coat, significant increases in the relative proportions of embryo weight and FA content in seeds, the alteration of the FA composition, and changes in the number and size of oil bodies in seed cells.

TT8 Controls Seed Starch and Protein Metabolism

The starch and protein contents of the wild-type (Col-0) and *tt8-4* seeds at 6 DAP, 16 DAP, and mature developmental stages were analyzed. As shown in Figure 3A, the starch contents of the wild type (209.38 ± 5.76 $\mu g\ mg^{-1}$ dry weight) and *tt8-4* (246.63 ± 6.37 $\mu g\ mg^{-1}$ dry weight) peaked in the developing seeds at 6 DAP. The mutant seeds exhibited significantly more starch than the wild-type seeds. The starch contents of both genotypes decreased considerably during the process of EM. In the seeds at 16 DAP and mature stages, only traces of starch could be detected in both the wild type and the mutant.

Figure 3B shows the increase of seed total protein contents from 6 DAP to the mature stage in the wild type and the *tt8-4* mutant. The seed protein content of the *tt8-4* seeds at 6 DAP was slightly higher than that of the wild type at the same stage; however, the *tt8-4* seeds at 16 DAP as well as at the mature stage contained lower protein than the wild-type seeds at the same stages. We also analyzed the amounts of proteins such as the 12S and 2S storage proteins in wild-type and *tt8* mutant seeds. Fractionation of identical amounts of protein extracts from wild-type and *tt8* mutant seeds by SDS-PAGE revealed quantity differences. As shown in Figure 4, the quantities of proteins, including 12S and 2S, in the *tt8* mutant seeds were uniformly lower than in the wild-type seeds; however, the overall proportions of individual proteins in the mutant were not significantly changed. Measurement of protein contents in mature seeds confirmed that the *tt8* seeds accumulated less protein than the wild-type seeds (Fig. 3B).

Effect of the *TT8* Mutation on Seed Coat Mucilage

Previous studies showed that TT8 regulates the biosynthesis of seed coat mucilage together with *ENHANCER OF GLABRA3* (Zhang et al., 2003). To

Table II. *Comparison of FA composition and total FA content (mol %) in mature seeds between tt8 mutant lines and their respective wild types*

Data reported are means of five independent experiments (five biological repeats) ± SE. The up or down arrows indicate an increase or decrease from the respective wild types.

Line	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	24:0
Ws	9.3 ± 0.8	3.3 ± 0.4	8.6 ± 0.4	36.6 ± 3.8	26.36 ± 3.53	1.6 ± 0.1	12.6 ± 0.6	0.3 ± 0.0	1.2 ± 0.4	0.3 ± 0.0
tt8-3	8.8 ± 0.8↓	2.9 ± 0.4↓	14.0 ± 0.5↑	31.9 ± 3.9↓	24.5 ± 4.5↓	1.9 ± 0.2↑	13.5 ± 0.6↑	0.4 ± 0.1↑	1.6 ± 0.3↑	0.5 ± 0.1↑
Col-0	9.8 ± 0.8	3.0 ± 0.3	6.5 ± 0.4	38.1 ± 3.6	27.2 ± 3.8	1.4 ± 0.2	12.6 ± 0.5	0.3 ± 0.1	1.0 ± 0.3	0.2 ± 0.0
tt8-4	9.3 ± 0.9↓	2.6 ± 0.5↓	14.0 ± 0.5↑	32.6 ± 4.0↓	24.9 ± 4.6↓	1.6 ± 0.2↑	13.0 ± 0.7↑	0.3 ± 0.1↑	1.4 ± 0.2↑	0.3 ± 0.1↑

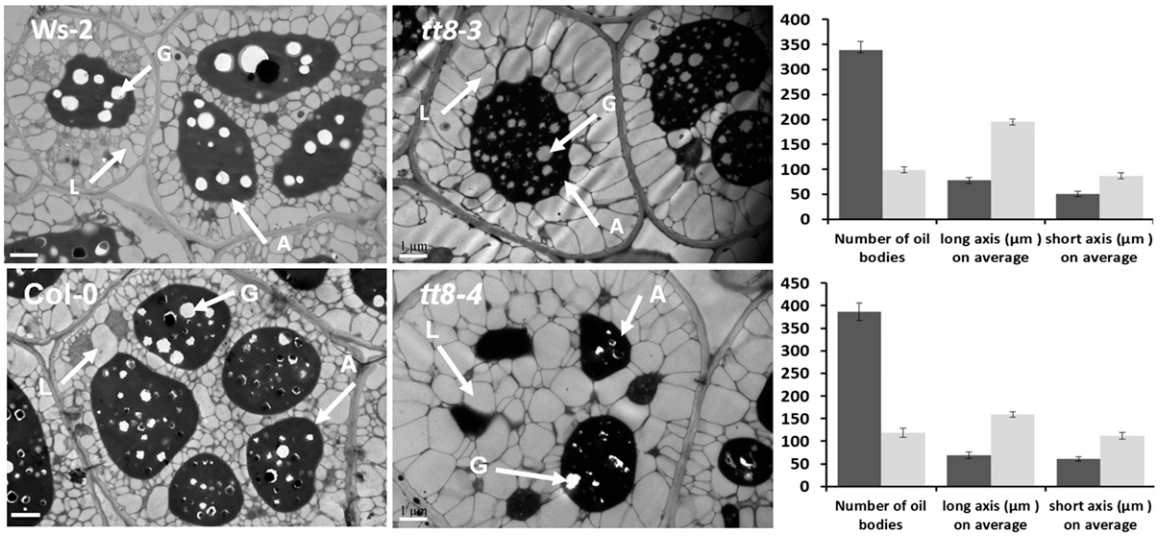

Figure 2. Subcellular differences in seed embryo cells between the wild types and *tt8* mutant lines in pairs. Arrows point to oil bodies (L), aleurone grains (A), and globoids (G). The quantitative data are given in charts beside each pair of mutant and wild-type images. Bars = 1 μm.

investigate if the *tt8* mutation resulted in less mucilage, we compared the mucilage layers between wild-type and *tt8* seeds. The result shows that there was no obvious difference in the thickness of mucilage layers between wild-type and *tt8* seeds (Fig. 5).

TT8 Acts Maternally to Influence Seed FA

Previous studies indicate that *TT8* regulates the biosynthesis of flavonoids in the seed coat, which is a maternal tissue. To understand whether *TT8* acts maternally or zygotically in affecting FA biosynthesis, we made reciprocal crosses between the wild type and the *tt8-4* mutant and measured the expression of *TT8* as well as FAs in the respective F1 seeds. Table III shows the homozygous or heterozygous status of the *TT8* gene in the embryo, endosperm, and seed coat tissues of various F1 hybrids derived from reciprocal crossings. In the F1 seeds derived from the crossings wild type × wild type and wild type × *tt8-4*, the levels of *TT8* expression were quite similar, peaking at 6 DAP and then decreasing at 10 and 14 DAP. In contrast, in the F1 seeds originating from the crossings *tt8-4* × wild type and *tt8-4* × *tt8-4*, the expression of *TT8* could hardly be detected (Fig. 6A). Moreover, high seed oil accumulations were measurable only in the F1 seeds from the crossings where *tt8-4* was used as a maternal parent, indicating that the *tt8* mutation had a maternal effect on seed FA accumulation (Fig. 6B).

TT8 Down-Regulates a Number of Genes Involved in Embryonic Development and FA Synthesis during Seed Development

The expression of the TFs regulating seed development, such as *LEAFY COTYLEDON1* (*LEC1*), *LEC2*,

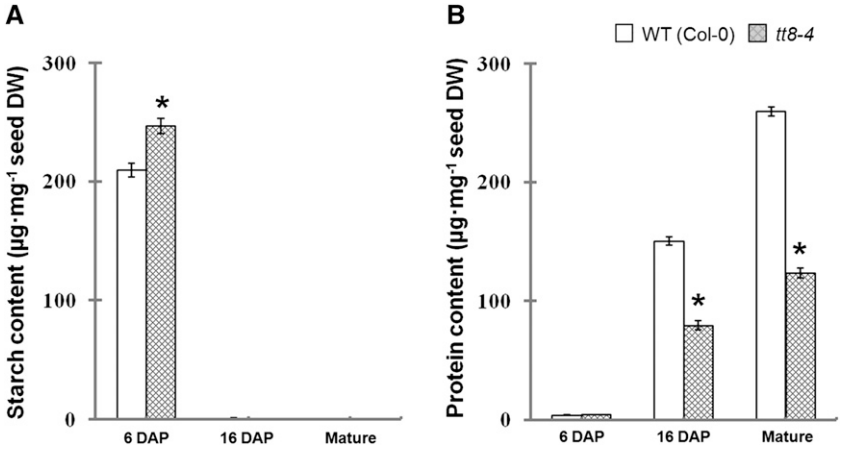

Figure 3. Evaluation of starch (A) and protein (B) contents in developing Arabidopsis seeds between the wild type (WT; Col-0) and the *tt8-4* mutant line. The starch and protein contents were analyzed at three different developmental stages, mature seeds and developing seeds at 6 and 16 DAP, based on the dry weight (DW) and seed total protein content. Data presented are means of three independent experiments (three biological repeats) ± SE. Asterisks indicate significant differences ($P \leq 0.05$) compared with the wild type.

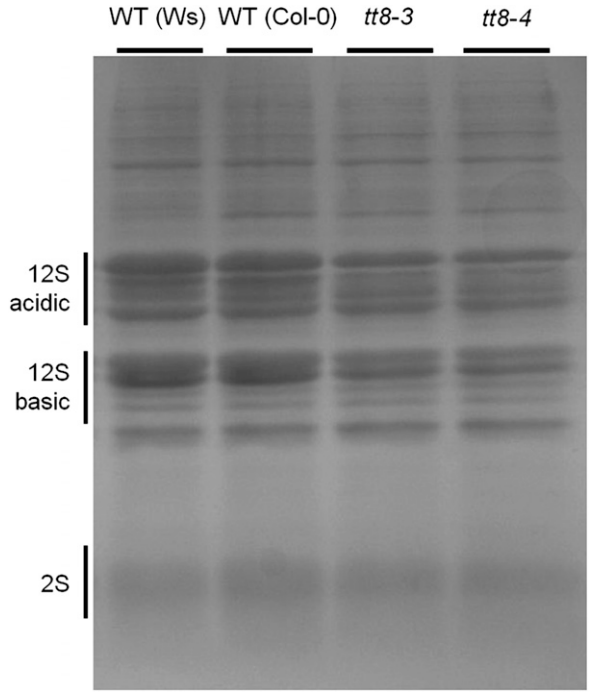

Figure 4. *tt8* mutant seeds contain less protein than do wild-type (WT) seeds. Protein extracts from an equal number of wild-type, *tt8-3*, and *tt8-4* mature seeds were fractionated on a 15% SDS polyacrylamide gel and stained.

and *FUSCA3* (*FUS3*), and the enzymes that are important in the FA biosynthesis pathway, including *ACETYL CO-ENZYME A CARBOXYLASE BIOTIN CARBOXYLASE SUBUNIT* (*CAC2*; At5g35360), *BETA-KETOACYL-ACP SYNTHETASE2* (*KASII*; At1g74960), *MOSAIC DEATH1* (*MOD1*; At2g05990), *FATTY ACID BIOSYNTHESIS2* (*FAB2*; At2g43710), *FATA ACYL-ACP THIOESTERASE* (*FatA*; At3g25110), *CYTIDINEDIPHOSPHATE DIACYLGLYCEROL SYNTHASE2* (*CDS2*; At4g22340), *FATTY ACID ELONGATION1* (*FAE1*; At4g34520), *FATTY ACID DESATURASE2* (*FAD2*; At3g12120), and *FAD3* (At2g29980), was investigated by performing RT-quantitative PCR (qPCR) using the RNAs extracted from seeds at 4, 6, 8, 10, 12, and 16 DAP (Fig. 7). Relative to the wild type, the *tt8* mutation resulted in about 2-fold higher *LEC1* and *LEC2* transcripts at early stages of seed development. The mutation did not significantly promote the relative expression of *FUS3* from 4 to 8 DAP but resulted in 3.5-fold higher transcripts at 10 DAP. As detailed in Figure 7, the *tt8-4* mutation did not lead to significant changes in the expression of genes encoding enzymes in the FA biosynthesis pathway at the developmental stages before 10 DAP but led to significant increases at 10 DAP and afterward. We also investigated the effect of *tt8-4* on the expression of *WRINKLED1* (*WRI1*) and *ABSCISIC ACID3* (*ABI3*), two other TFs regulating seed development, and of *CAC3* (At2g38040), *BCCP2* (At5g15530), and *PIS2* (At4g38570), other enzymes in the FA biosynthesis pathway. However, there were no significant expression differences in these genes between the wild type and *tt8-4* (Supplemental Fig. S4). Taken together, the *tt8-4* mutation promoted a class of genes regulating either embryonic development or FA synthesis during seed development.

TT8 Directly Targets *LEC1*, *LEC2*, *FUS3*, and *CDS2* in Arabidopsis Seedlings

To further elucidate whether the TFs and enzymes mentioned above are the direct downstream targets of TT8, an inducible *TT8* gene fused with the steroid-binding domain of glucocorticoid receptor (*35S:TT8-GR*) was constructed and transferred into the wild type and then crossed with *tt8-4* mutant plants. Treatment of *tt8-4* plants harboring *35S:TT8-GR* 20 d after sowing with the mammalian steroid hormone analog dexamethasone (DEX) rescued the yellow seed coat phenotype of the *tt8-4* mutant (Supplemental Fig. S5). Thus, DEX treatment induced high levels of TT8 activity. RT-qPCR was performed using the RNA samples from the transgenic seedlings (the *tt8-4* plants harboring the *35S:TT8-GR* construct) 15 d after sowing mock treated or treated with 10 μM DEX or 10 μM DEX plus 5 μM cycloheximide (CYC) for 1, 4, 7, and 10 h. Figure 8 shows that DEX treatment for 1 h or longer resulted in continuous reduction of *LEC1*, *LEC2*, *FUS3*, and *CDS2* RNA levels relative to the mock-treated controls. To determine whether 35S:TT8-GR directly or indirectly represses these four genes, we repeated DEX applications to the *35S:TT8-GR* seedlings of the *tt8-4* background in the presence of the protein synthesis inhibitor CYC, because the induction of TT8-GR activity by DEX does not require protein synthesis. The results showed that CYC strongly removed the repression

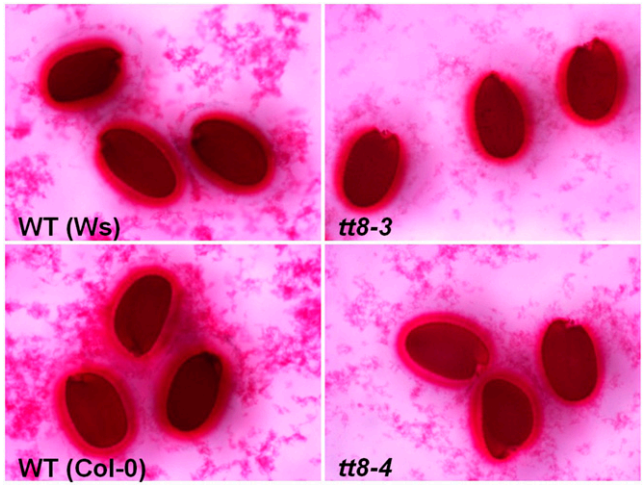

Figure 5. Comparison of the mucilage layer attached to the seed coat between wild-type (WT) and *tt8* mutant seeds. [See online article for color version of this figure.]

Table III. *Status of the TT8 allele in the embryo, endosperm, and seed coat tissues of F1 seeds derived from Col-0, tt8-4, and the reciprocal crossings between Col-0 and tt8-4*

Plus and minus signs indicate dominant *TT8* and recessive *tt8* status, respectively.

Tissue	Col-0 × Col-0	Col-0 × *tt8-4*	*tt8-4* × Col-0	*tt8-4* × *tt8-4*
Embryo	++	+–	–+	–
Endosperm	+++	++–	–+	–
Seed coat	++	++	–	–

of these genes exerted by DEX treatment, especially at 4 and 7 h after combined treatment with DEX and CYC (Fig. 8), suggesting that *LEC1*, *LEC2*, *FUS3*, and *CDS2* could be the direct targets of TT8.

To further elucidate the molecular mechanism by which TT8 affects the expression of *LEC1*, *LEC2*, *FUS3*, and *CDS2*, chromatin immunoprecipitation (ChIP) assays were performed to test whether TT8 could bind to the promoter regions of these four genes using *35S*:*TT8-6HA* transgenic plants (*HA* for *human influenza hemagglutinin epitope*) in which yellow seed coat was restored (Supplemental Fig. S5). The TT8 protein displays the typical features of a TF, with a basic helix-loop-helix signature at its C terminus, which recognizes the binding consensus site CANNTG (Abe et al., 1997; Massari and Murre, 2000). The abundance of the TT8-6HA protein in nuclear extracts (input) of *35S*:*TT8-6HA* rosette leaves and siliques and the corresponding immunoprecipitated fractions (eluate) used for ChIP assays was comparable to that in the wild type after fixation (Fig. 9A). ChIP assays showed that TT8-6HA was associated with the promoter regions near fragments 1, 3, and 4 of *LEC1*, fragments 2, 3, and 6 of *LEC2*, fragments 6, 7, 8, and 9 of *FUS3*, and fragments 1 and 3 of *CDS2*. Additionally, fragments 4, 3, 8, and 3 of *LEC1*, *LEC2*, *FUS3*, and *CDS2*, respectively, showed the highest enrichment folds (Fig. 9, B and C). These data strongly support the hypothesis that these four genes are directly targeted by TT8.

DISCUSSION

The factors and the regulatory network that control FA biosynthesis in plant seeds are still largely unknown and require further investigation. In this study, we discover the novel function of *TT8* in regulating seed FA accumulation in Arabidopsis. We demonstrate that the *tt8* mutation drastically increases seed FA content (about 64%; Tables I and II; Fig. 1C), in particular the seed embryo FA content (Fig. 1D), and also changes the proportion of various FA species (Tables I and II). The significant increase of FA content in the *tt8* seeds was accompanied by reductions of seed coat proportion (Fig. 1B) and protein deposition in embryos (Figs. 3B and 4) and by an elevation of starch synthesis at early seed developmental stages (Fig. 3A). The *tt8* embryos are heavier than wild-type embryos, reflecting the differences in developmental effects from metabolic outcomes. *TT8* acts maternally to affect seed FA accumulation (Fig. 6). Furthermore, it directly binds to the regulatory regions of *LEC1*, *LEC2*, *FUS3*, and *CDS2* and down-regulates the expression of these genes (Figs. 7–9). On the other hand, it indirectly inhibits the expression of a number of genes, including *CAC2*, *KASII*, *MOD1*, *FAB2*, *FatA*, *FAE1*, *FAD2*, and *FAD3*, that catalyze various steps in the FA biosynthesis pathway (Fig. 6).

In Arabidopsis, *TT8*, together with *TT2* and *TTG1*, synergistically regulates flavonoid biosynthesis (Nesi et al., 2000, 2001; Debeaujon et al., 2003; Lepiniec et al., 2006). The reduction of flavonoids in the seed coat caused yellowish *tt8* seeds (Nesi et al., 2000; Supplemental Fig. S2), a reduction of seed coat weights, and heavier embryos based on similar seed weight (Fig. 1, A and B). Consequently, mature *tt8-4* seeds yield higher FAs in seeds and embryos (Fig. 1, C and D). Nevertheless, there were no obvious differences in the sizes of embryo cells, embryos (Supplemental Fig. S3), and seeds (Supplemental Fig. S1) between the wild type and *tt8* mutants.

In this study, we observed that *tt8* mutants have larger oil bodies (Fig. 2). However, it was difficult to determine a causal relationship between oil body size/number and the oil content. In one of our previous publications, we analyzed the possible functions of a group of lipases, named seed FA reducers, to diminish

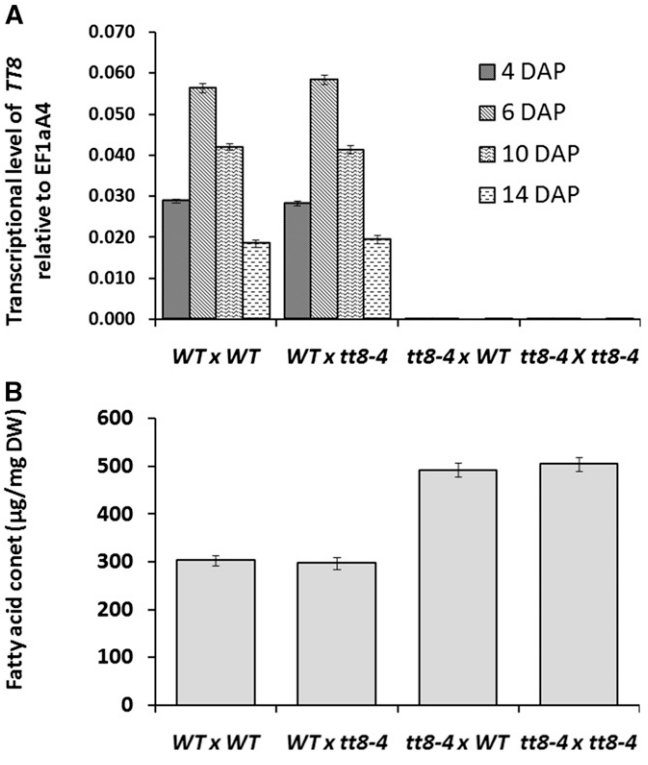

Figure 6. Maternal effects of the *tt8-4* mutation on *TT8* expression in embryonic tissues (A) and seed FA accumulation (B). DW, Dry weight; WT, wild type.

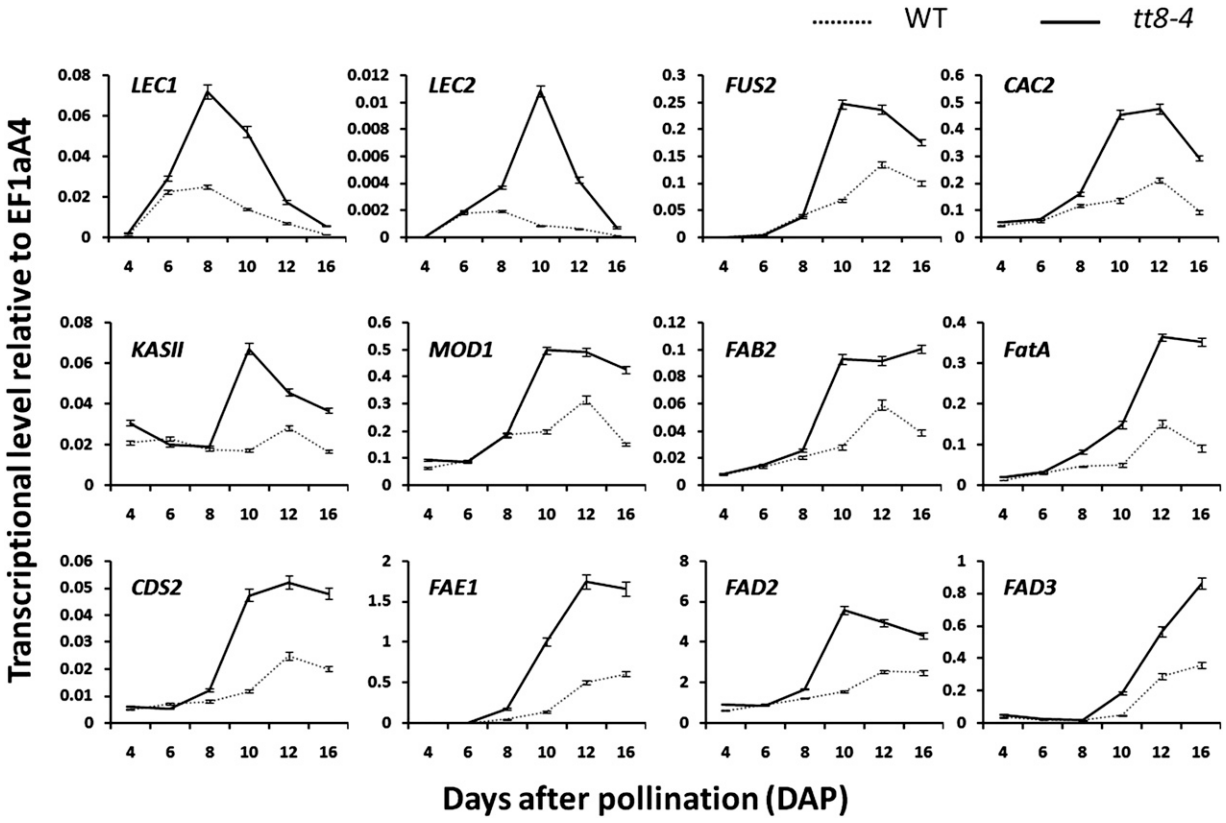

Figure 7. Comparison of the transcription levels of genes either regulating seed development or important in the FA synthesis pathway at different developmental stages between wild-type (WT) and *tt8-4* seeds. RNA samples were extracted from the developing seeds at 4, 6, 8, 10, 12, and 16 DAP. The gene transcripts were measured by RT-qPCR using *EF1aA4* (At5g60390) as the internal standard. Values are means of two replicates carried out on cDNA dilutions obtained from two independent RNA extractions.

the size of oil bodies by hydrolyzing triacylglycerol (Chen et al., 2012a). We also noticed that large oil bodies sometimes are correlated with lower seed oil content. For example, the disruption of *OLEOSIN* by RNA interference results in unusually larger oil bodies that correlate with lower seed lipid content (Siloto et al., 2006). Moreover, some rapeseed (*Brassica napus*) cultivars with low oil content have oil bodies similar in size to those in cultivars with high oil content (Hu et al., 2009).

Seed starch accumulates transiently during EM in Arabidopsis (Baud et al., 2002). Some studies proposed that starch accumulation strengthens a seed as a principal sink before EM starts, and starch degradation serves the synthesis of storage compounds after EM ends (Norton and Harris, 1975; daSilva et al., 1997; Periappuram et al., 2000). By contrast, seed protein is deposited mainly during the seed maturation stage, reflected by the high expression of a class of genes encoding the seed storage proteins in embryos (Pang et al., 1988; Baud et al., 2002). The storage proteins of Arabidopsis seeds consist mainly of a 12S globulin (cruciferin) and a 2S albumin (arabin; Heath et al., 1986). It is believed that the patterns of starch and/or protein accumulation in developing seeds affect seed FA biosynthesis remarkably (Mu et al., 2008; Chen et al., 2012b). Our study demonstrates that the amount of seed starch accumulation at 6 DAP is negatively correlated with the amount of protein deposition at late stages (16 DAP or the mature phase; Figs. 3 and 4), suggesting that the *tt8-4* mutation supplies more carbohydrates for the biosynthesis of FAs, which otherwise may be used for the production of proteins.

Seed mucilage production in Arabidopsis is part of a remarkable differentiation process during which the epidermal cells of the mature ovule grow, rearrange their cytoplasm, synthesize and secrete mucilage, and form a secondary cell wall (Western et al., 2000). Several mutants associating with abnormal seed coat development, including *tt2* and *gl2*, demonstrated an elevated seed FA content but a reduced mucilage layer in comparison with wild-type plants (Shi et al., 2012). It is supposed that seed coat mucilage and seed FAs are the competing sinks for the limiting photosynthates imported into developing seeds (Shi et al., 2012). In our study, we did not observe a significant reduction in mucilage layer in *tt8-4* seeds (Fig. 5), suggesting that the *tt8-4* seeds yield higher FAs without any cost in mucilage biosynthesis.

The comparison of FAs in the F1 seeds generated from reciprocal crossings between wild-type and *tt8-4*

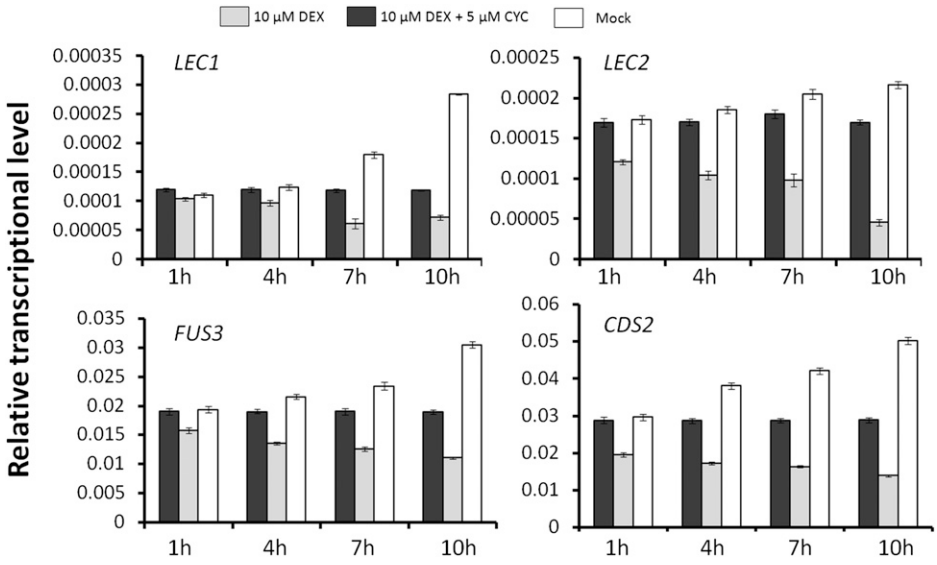

Figure 8. Induced TT8 activity can transcriptionally repress the expression of *LEC1*, *LEC2*, *FUS3*, and *CDS2*. RT-qPCR results were determined from RNA samples of *tt8-4 35S:TT8-GR* seedlings 15 d after sowing mock treated or treated with 10 μM DEX or 10 μM DEX plus 5 μM CYC for 1, 4, 7, and 10 h. The transcriptional level of the mock-treated samples at each time point was set to 1, and *EF1aA4* was used as the internal control. Values are means of three biological repeats, and error bars indicate SE.

plants indicates a clear maternal effect of the *TT8* function. Only the F1 embryos derived from a crossing in which *tt8-4* was used as the maternal parent exhibited a higher FA yield (Fig. 6). There could be a close association between the yield of FA in embryonic tissues and the amount of flavonoids in the surrounding seed coat, because flavonoids are considered to be inhibitors of FA biosynthesis, repressing the expression of *FabG* and *FabI*, which encode important reductases involved in chain elongation during FA biosynthesis (Zhang and Rock, 2004). They could possibly permeate from a seed coat to an adhering embryo, inhibiting the accumulation of FAs in the embryonic tissues. Another interpretation could be an epigenetic modification such as gene imprinting, since it seems that the expression of the *TT8* allele demonstrated a clear parent-of-origin effect (i.e. that the *TT8* allele derived from the paternal parent was not expressed; Fig. 6A; Grossniklaus et al., 1998; Adams et al., 2000; Gehring et al., 2004; Jiang and Köhler, 2012).

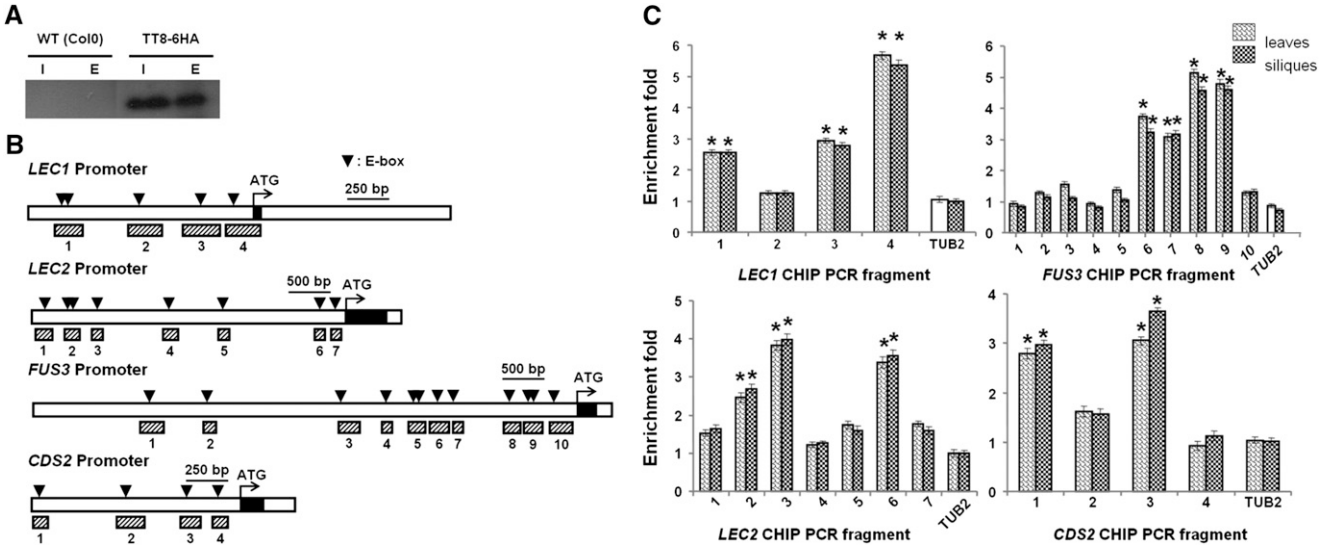

Figure 9. ChIP enrichment test showing the binding of TT8-6HA to the *LEC1*, *LEC2*, *FUS3*, and *CDS2* promoters. A, Western-blotting results showing the TT8-6HA band. I, Input, E, eluate. B, The promoter structures of *LEC1*, *LEC2*, *FUS3*, and *CDS2*. The upstream region and the first intron of the genes are represented by white boxes, while the first exon is represented by the black box. The arrowheads at the top indicate the sites containing putative E-boxes on the promoters of these four genes above. Hatched boxes represent the DNA fragments amplified in ChIP assays. C, ChIP assay results in rosette leaves and siliques. *EF1aA4* was used as an internal control. Significant differences in comparison with the enrichment of a *TUBULIN BETA CHAIN2* (*TUB2*) fragment are indicated with asterisks. Error bars denote SE.

Seed FA biosynthesis starts by the formation of malonyl-CoA from the precursor acetyl-CoA catalyzed by acetyl-CoA carboxylase (ACCase), which is the key switch that controls FA flux, acting as a sensor or a gating system to monitor the overall flux of FA biosynthesis (Mu et al., 2008). ACCase is composed of three subunits, CAC2, CAC3, and BCCP2. *CAC2* was up-regulated in developing *tt8-4* seeds at 10 and 12 DAP (Fig. 7). More active ACCase should lead to an elevation of FAs in the *tt8* seeds. On the other hand, the genes catalyzing the rest of the critical steps in the FA synthetic pathway, such as *KAS* (which transfers acetyl-CoA and malonyl-ACP to 3-ketoacyl-ACP), *MOD1* (which encodes an enoyl-ACP reductase), *FAB*2 (which is a stearoyl-ACP desaturase), *FatA* (which encodes acyl-ACP thioesterase; Moreno-Pérez et al., 2012), *CDS2* (which catalyzes 1,2-diacylglycerol-3-phosphate into CDP-diacylglycerol), *FAD*2 (which serves for the synthesis of polyunsaturated lipids; Okuley et al., 1994), and *FAD3* (which is essential for the formation of the FA 18:3; Shah et al., 1997), were also moderately up-regulated at various stages and drastically peaked at 10 DAP in the *tt8* developing seeds (Fig. 7). These up-regulated genes would be responsible for the elevated seed FA accumulation and the changed proportions of various FA species in the *tt8* seeds.

FA accumulation is determined at multiple levels, of which transcriptional regulation is essential for controlling the FA biosynthesis pathway (Ohlrogge and Jaworski, 1997; Millar et al., 2000). The key TFs controlling FA accumulation mainly include *WRI1*, *LEC1*, *LEC2*, *FUS3*, and *ABI3*, which have a large number of downstream target genes. In Arabidopsis, *LEC1* shares extensive sequence similarity with the HAPLESS3 subunit of a CCAAT-binding TF, and the increased expression of the gene elevates many FA biosynthetic genes involved in condensation, chain elongation, and desaturation (Lee et al., 2003; Mu et al., 2008). *LEC2* and *FUS3* are B3 domain TFs that play essential roles in the regulation of seed maturation (Luerssen et al., 1998; Stone et al., 2001; To et al., 2006). The LEC2 protein induces many maturation traits, including auxin activity, and the expression of other seed development regulators such as *FUS3* and *ABI3*, which function redundantly in many aspects, including seed storage protein accumulation, chlorophyll degradation, and abscisic acid responses (Luerssen et al., 1998; To et al., 2006; Baud et al., 2007). These TFs function in unique and overlapping ways in regulating embryonic development in Arabidopsis (Wang et al., 2007; Santos-Mendoza et al., 2008; Baud and Lepiniec, 2009). In this study, the *tt8* mutation did not cause significant changes in the expression levels of *WRI1* and *ABI3* (Supplemental Fig. S4) but did cause significant changes in the expression levels of *LEC1*, *LEC2*, and *FUS3*, in particular, a remarkable increase of *FUS3* expression at the late stage of seed development (Fig. 7), which was consistent with the *TT8* expression pattern (Nesi et al., 2000; Xu et al., 2013).

By posttranslational activation of TT8-GR, we further demonstrated the repression of *LEC1*, *LEC2*, *FUS3*, and *CDS2* by induced TT8 activity (Fig. 8). Moreover, down-regulation of these four genes by DEX treatment of TT8-GR seedlings was not affected by CYC, indicating that the repression of these four genes by TT8 is independent of protein synthesis (Fig. 8). ChIP assays using specific HA antibodies further revealed in vivo TT8 binding to the cis-regulatory regions of these genes (Fig. 9), thus suggesting that TT8 acts as a direct regulator repressing *LEC1*, *LEC2*, and *FUS3*, which have a large number of downstream targets during seed development, and *CDS2*, which mediates glycerolipid biosynthesis.

Taken together, we show that the *tt8* mutation remarkably increased the FA contents of seeds, altered the FA composition, and lowered seed protein accumulation during seed maturation. TT8 acted maternally to affect seed FA accumulation, and it repressed the expression of *LEC1*, *LEC2*, *FUS3*, and *CDS2* by binding to the cis-regulatory regions of these genes and indirectly down-regulated a number of other genes, such as *CAC2* (At5g35360), *KASII* (At1g74960), *MOD1* (At2g05990), *FAB2* (At2g43710), *FatA* (At3g25110), *CDS2* (At4g22340), *FAE1* (At4g34520), *FAD2* (At3g12120), and *FAD3* (At2g29980), which encode enzymes catalyzing various steps of FA biosynthesis. For decades, yellow-seeded *Brassica* species oilseeds have been interesting to breeders. This work enriches our knowledge of why yellow seeds accumulate more FAs in Arabidopsis, a model plant belonging to the family Cruciferae that includes major *Brassica* species oilseeds.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All the transgenic Arabidopsis (*Arabidopsis thaliana*) plants are of the Col-0 background. *tt8-3* and *tt8-4* are of the Ws and Col-0 backgrounds, respectively. The primers used for the *tt8-4* genotyping are listed in Supplemental Table S1. The Arabidopsis plants were grown with a light cycle of 16 h of light and 8 h of dark. The light tubes were turned off at 11 PM and turned on at 7 AM, and the tissue samples were collected around 1 PM. Other growth conditions are given in our previous publications (Chen et al., 2012a, 2012b).

Dissection of Seeds

For the separation of seed coat from embryo, we soaked the seeds in tap water at 4°C for at least 1 h and then carefully detached the embryos from seed coats using a pair of forceps. The seed coats actually came off with endosperm layers. The detached embryos were further observed using a microscope to determine their sizes and the cell shapes/cell numbers of the epidermal layer. The seed coats were dried at room temperature and then weighed.

Determination of FAs, Seed Starch, and Protein Content

The extraction and analysis of FAs in the seed and seed embryo were carried out as described before (Poirier et al., 1999; Chen et al., 2012b). The quantification of the seed starch and protein was determined as described previously by Chen et al. (2012b).

Microscopy Analysis

To observe the seed coat color, mature seeds of the wild type and *tt8* mutants were randomly selected and photographed using an Olympus SZ 61 stereomicroscope. The seeds stained with Ruthenium Red were inspected with

a compound light microscope (Nikon SMZ1500) and photographed. The embryo size and embryo cell size were measured as follows (Ohto et al., 2005). Twenty mature dried seeds of each line were imbibed for 1 h and dissected using the microscope to isolate mature embryos. Embryos were incubated in a buffer (50 mM sodium phosphate, pH 7, 10 mM EDTA, 1% [v/v] Triton X-100, and 1% [v/v] dimethyl sulfoxide) at 37°C overnight, fixed with 10% (v/v) formalin, 5% (v/v) acetic acid, 45% (v/v) ethanol, and 0.01% (v/v) Triton X-100 for 45 min, and then rehydrated using a graded series of ethanol (50%, 70%, 80%, 90%, 95%, and 100% [v/v]) for about 5 min each. Embryos were then treated for 1 h in Hoyer's solution (chloral hydrate:water:glycerol, 3:0.8:0.4). The cleared embryo and embryo cell sizes were observed and measured using an Olympus compound microscope (Nikon SMZ1500) with differential interference contrast optics. Transmission electron microscopy observation was performed as described previously (Chen et al., 2012a, 2012b).

Protein Analysis by SDS-PAGE

We used the method to analyze different kinds of seed total protein described by Ohto et al. (2005). In brief, 15 mature dried seeds from wild-type plants or *tt8* mutants were homogenized with 30 μL of extraction buffer (100 mM Tris-HC1, pH 8, 0.5% [v/v] SDS, 10% [v/v] glycerol, and 20% [v/v] 2-mercaptoethanol) using a microglass pestle and mortar, followed by boiling for 5 min and centrifugation for 10 min at highest speed at 4°C (Naito et al., 1988). Fifteen microliters of each extract was used for SDS-PAGE.

Ruthenium Red Staining for Seed Coat Mucilage

Dry seeds from each line were randomly selected and shaken in water for 1 h and then stained in a 0.01% (w/v) Ruthenium Red solution.

RT-PCR and RT-qPCR

Flowers on primary shoots were tagged with different colored threads to indicate the days after pollination, and the seeds from the tagged siliques were harvested for RNA extraction. Young leaves at the rosette stage were utilized for RNA extraction to identify the transgenic plants. The total RNA of different tissues was isolated using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized in a 10-μL reaction solution containing about 1.5 μg of total RNA reverse transcribed by the ThermoScript RT-PCR system (Invitrogen). Diluted aliquots of the reverse-transcribed cDNAs were used as templates in RT-qPCR containing the SYBR Green PCR Master Mix (Applied Biosystems). RT-qPCR assays were performed in triplicate on the 7900HT Fast Real-Time PCR system (Applied Biosystems). All the primers for the semiquantitative RT-PCR and RT-qPCR analysis are listed in Supplemental Table S2.

Generation of Transgenic Plants

To construct *35S:TT8-6HA*, the *TT8* cDNA was amplified with the primers TT8_P1_*Xho*I (5′-ATTCTCGAGATGGATGAATCAAGTATTATTCC-3′) and TT8-6HA_P2_*Spe*I (5′-GGACTAGTTAGATTAGTATCATGTATTAT-3′). The PCR products were digested by *Xho*I and *Spe*I and then cloned into pGreen-35S-6HA to obtain an in-frame fusion of *TT8-6HA* under the control of the 35S promoter (Liu et al., 2008). Similarly, the *TT8* cDNA was also amplified with the primers TT8_P1_*Xho*I and TT8-GR_P2_*Cla*I (5′-CCATCGATTAGATTA GTATCATGTATTAT-3′) and then cloned into pGreen-35S-GR to obtain an in-frame fusion of *TT8-GR* under the control of the 35S promoter (Yu et al., 2004). These constructs were transformed into *Agrobacterium tumefaciens* strain GV3101, which was then used for the transformation of Arabidopsis wild-type (Col-0) plants via floral dip (Clough and Bent, 1998). The transgenic plants were selected by basta until the T3 homozygous transgenic plants were generated, and then RT-qPCR and western blotting were applied to further verify the transgenic plants. The confirmed *35S:TT8-6HA* and *35S:TT8-GR* transgenic plants were independently crossed with *tt8-4* to check whether the yellow seed coat of *tt8-4* can be rescued in the two transgenic plants.

GR Induction

For the induction of *TT8:GR*, seedlings at 15 d after sowing were collected 1, 4, 7, and 10 h after a single mock, DEX, or DEX plus CYC treatment. The concentrations of DEX and CYC were 10 and 5 μM, respectively.

ChIP Assay

The ChIP assays were performed according to Xi et al. (2010). For each ChIP assay, three independent experiments were performed using rosette leaves or siliques collected separately. DNA enrichment was examined by real-time qPCR in triplicate as described previously (Liu et al., 2008). The enrichment of a TUB2 genomic fragment was used as a negative control. Primer pairs used for the ChIP enrichment test are listed in Supplemental Table S3.

Statistical Analysis

A completely randomized block design with at least three biological replicates for each experiment was applied. The relative amounts of RNAs were calculated using the method of Livak and Schmittgen (2001). Data were classified with Win-Excel and analyzed via ANOVA using the SPSS (version 8.0) statistical package. Comparisons between the treatment means were made using Tukey's test at $P \leq 0.05$.

Supplemental Data

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

Supplemental Figure S1. Identification of *tt8* mutant lines.

Supplemental Figure S2. Microscopic observation of *tt8* mature seeds.

Supplemental Figure S3. The embryo size and cell size are similar to those of the wild type.

Supplemental Figure S4. Comparison of the transcription levels of *WRI1*, *ABI3*, *CAC3*, and *BCCP2* genes at different developmental stages between wild-type and *tt8-4* seeds.

Supplemental Figure S5. Confirmation of the functional transgenic plants of *35S:TT8-GR* and *35S:TT8-6HA*.

Supplemental Table S1. Primers used for genotyping of the *tt8-4* mutants.

Supplemental Table S2. Primers used for RT-PCR and RT-qPCR analyses.

Supplemental Table S3. Primers used for the ChIP assay.

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